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CONCENTRATION OF IODINE IN THE HUMAN STOMACH AND OTHER TISSUES AS DETERMINED WITH RADIOACTIVE IODINE

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ANIMAL tissues other than the thyroid are not commonly known to concentrate iodine, although high concentrations of iodine in saliva and urine have been observed repeatedly. Chaikoff has said that the tissue distribution of iodine after large doses of iodide "suggests a diffusion of iodine in and out of the tissues rather than a selective retention,"[1] a point of view also expressed by others.[2, 3, 4, 5] However, it has been noted that levels of iodine in the gastric juice are several times higher than the simultaneous serum iodide levels in man,[6] and in pouch dogs[7] after giving ordinary iodide, and in man[8] and fetal animals[9] after giving radioactive iodide. Chloride in the gastric juice, in contrast, never rises much above the serum chloride concentration.[10]

In this investigation, the distribution of iodine injected as iodide was measured in the tissues of patients later coming to autopsy to learn if (a) human stomach tissue concentrates iodine above serum values, and if (b) distribution of iodine among tissues is affected by variations in the quantity of iodine given and in the period elapsing between iodine administration and death.

The use of radioactive iodine* permitted us to follow the injected iodine as differentiated from the iodine already present in the body at the time of injection. The tissue distribution of the injected iodine was found to be different from the data published for chlorides[11]; therefore analysis of these tissues for chloride was also performed.

METHODS

Fourteen patients who later came to autopsy were given iodine injections. The expectation of autopsy and the absence of iodine-containing medications were the only criteria used in selection of these patients. Therapy was not altered for purposes of this investigation. All fourteen subjects received 100 to 500 μc† of I^{131}; five received 75 to 300 mg. of inert iodine added as sodium iodide; six received 0.4 to 2.6 μg of inert iodine added as sodium iodide; and three received no additional iodine, only "carrier-free" I^{131}. Sterile

From the Gastric Laboratory of the Department of Internal Medicine, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital.

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*The radioactive iodine, I^{131}, used in this investigation was supplied by the Clinton Laboratories, Oak Ridge, Tenn., and was obtained on allocation from the United States Atomic Energy Commission.

†All absolute values for radioactivity are based solely upon the latest figures given by the supplier.

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TABLE I. RATIOS OF I^{131} CONCENTRATION PER GRAM OF FRESH TISSUE TO THE I^{131} CONCENTRATION LABELED

SUBJECT	AUTOPSY DIAGNOSIS	TIME BETWEEN IODINE ADMINISTRATION AND DEATH (HR.)	QUANTITY OF IODINE INJECTED	QUANTITY OF INJECTED IODINE FOUND PER LITER OF SERUM	UPTAKE OF INJECTED IODINE BY THYROID (% OF DOSE)
C. S.	Hypostatic pneumonia	4½	163 mg.	3.1 mg.	0.1
K. B.	Cerebral venous thrombosis	6	170 mg.	6.9 mg.*	0.1
E. G.	Cerebral venous thrombosis	7½	170 mg.	3.4 mg.	0.1
W. S.	Carcinoma of the prostate	55	155 mg.	12.0 mg.	5.3
E. R.	Bronchopneumonia; toxic nephrosis	78	170 mg.	8.4 mg.	1.5
F. S.	Accelerated arteriolonephrosclerosis	3	2.6 μg	0.06 μg	1.3
C. K.	Mitral valvulitis	3	1.3 μg	0.16 μg	0.8
T. W.	Myocardial infarction	5	1.6 μg	0.06 μg	1.4
M. P.	Benign gastric ulcer	12	1.3 μg	0.09 μg	0.8
F. R.	Accelerated arteriolonephrosclerosis	98	0.4 μg	0.03 μg	16.0
F. D.	Accelerated arteriolonephrosclerosis	159	1.7 μg	0.04 μg	1.1
A. F.	Lipoid pneumonia; pleural carcinoma	4	0.004 μg	0.000,2 μg	10.0
F. W.	Intraventricular hemorrhage	118	0.002 μg	0.000,005 μg	36.0
E. K.	Carcinoma of the gall bladder with metastases	156	0.004 μg	0.000,03 μg	58.0

*A ratio of 0.04 for cerebrum I^{131}/serum I^{131} was found in this subject.

sodium iodide and the I^{131} were made up to 10 ml. with sterile water and injected intravenously. A Geiger counter revealed no great loss of radioactivity on the glassware used in preparing and administering these labeled sodium iodide solutions.

Autopsies were performed within a twelve-hour period following death. The thyroid and portions of the tissues noted in Tables I and II were removed, weighed, and placed in 1 per cent sodium hydroxide. Samples of 5 to 15 grams of tissue were taken to ensure representative sampling. The technique of preparing tissues for measurement of radioactivity was adapted from the method used by Keating and associates[12] to measure the radioiodine content of chick thyroids. The tissues in 1 per cent sodium hydroxide were warmed to 30 to 50° C. and shaken. Homogeneous suspensions were thus obtained, fine enough to permit pipetting. One milliliter of such a suspension represented 100 mg. of fresh tissue and 900 mg. of 1 per cent sodium hydroxide. This simple procedure minimizes handling and transfer of samples by avoiding ashing, extraction, or distillation procedures[12-16] and has proved efficient in measurements on several thousands of tissue samples.

For measurement of radioactivity 0.2 ml. aliquots of tissue suspensions or 0.1 ml. of serum were pipetted onto No. 2 microscope cover glasses 22 mm. diameter and allowed to dry, sometimes with the help of an infrared lamp. These cover glasses were placed on plastic holders below Victoreen mica-window Geiger-Müller counters in such a way as to obtain a geometrical efficiency of 20 to 25 per cent. The counters, shielded with 2 to 3 in. of lead, had windows 29 mm. in diameter and 3 to 5 mg. per square centimeter thickness, and were connected to scalers with circuits of Higinbotham's design and scales of 1 to 64.

Cover glasses were also prepared, as standards with which to compare other materials, from water dilutions of the solutions of I^{131} obtained from Oak Ridge. A few milligrams of sodium iodide had first been added to the contents of the containers from Oak Ridge to avoid possible loss of I^{131} on glass surfaces.[18] Aliquots were pipetted onto the cover glasses, mixed there with a drop of silver nitrate solution (0.5 mg. per milliliter), and air dried. Cover glasses had to be well cleaned to permit even spreading of the aqueous suspension.

PER MILLILITER OF SERUM IN FOURTEEN SUBJECTS FOLLOWING ADMINISTRATION OF RADIOACTIVELY IODIDE

THYROID	STOMACH TISSUE			KIDNEY	LIVER	LUNG	HEART	MUSCLE	SPLEEN	ADRENAL	SKIN
	CARDIA	FUNDUS	PYLORUS								
SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM
2.0	1.1	1.1	1.1	0.6	0.5	0.9	0.4	0.3	0.6	0.7	–
1.4	3.2	4.6	2.2	0.5	–	0.7	0.4	0.2	0.5	0.8	5.8
3.5	1.4	2.0	1.1	0.7	0.7	1.3	0.6	0.4	0.7	2.3	0.8
8.0	2.6	3.7	3.3	0.6	0.5	0.2	0.3	0.5	0.6	–	0.4
25.	5.4	2.9	5.1	0.9	0.3	1.2	0.7	0.7	0.6	0.9	0.7
15.	–	–	8.0	0.7	0.8	0.7	0.4	0.4	0.7	0.8	–
5.0	0.1	–	–	0.3	0.3	–	–	–	0.3	0.3	–
20.	–	–	–	–	–	–	–	–	–	–	–
6.0	0.7	0.9	0.7	0.7	0.7	–	–	0.5	0.8	0.6	–
186.	6.7	6.7	3.0	0.8	0.9	0.2	0.0	1.3	1.3	0.8	1.3
20.	5.0	1.8	0.5	1.4	1.0	1.7	0.6	0.3	2.0	0.8	0.5
39.	2.0	2.0	2.0	0.7	0.3	–	–	0.3	2.0	0.8	0.5
5800.	4.0	2.0	2.0	1.6	1.7	–	–	2.1	1.9	0.0	0.9
3100.	3.3	3.3	5.3	0.8	0.6	–	–	0.5	1.1	0.8	0.6

These standards frequently, but not always, lost a portion of their radioactivity, 2 to 8 per cent in a week's time, other than the loss due to radioactive decay. At the same time the material on the cover glasses blackened, suggesting the release of free silver and of iodine. New cover glasses had to be prepared from fresh aliquots of the standard solution by this time and were always found to give the expected eight-day half-life when compared with data obtained earlier on other freshly prepared cover glasses. (The comparison was facilitated by use of samples of RaD and E, kindly furnished by Dr. L. F. Curtiss of the Bureau of Standards, through which corrections for changes in the sensitivity of the counting equipment could be made.) Standards were always counted within two hours of the time any sample aliquot was counted. The radioactivity of tissue aliquots was measured until at least 400, often 800 or more, impulses above background (15 to 20 counts per minute) were counted. Background was always counted at least as long as any sample was counted. Further details of the analytic technique are available in a recent publication.[19]

Measurements were expressed as ratios between the rates of counting of the tissue aliquots and of the standards. Since the quantity of radioactivity accompanying a given quantity of injected iodine was known, these ratios could be used to calculate the quantity of injected iodine present per kilogram of fresh tissue or per liter of serum. No corrections were made for self-absorption of beta rays in the dried aliquots of tissues, serum, or standards, thus introducing an error of several per cent into the results. This method of calculation assumes that the I^{131} is in no way separated from the inert iodine simultaneously administered, an assumption considered valid in radioactive tracer work with heavier atoms such as these. It is also assumed that the inert iodine and the radioactive iodine are in the same chemical form, chiefly iodide in this instance, an assumption which may sometimes be invalidated by the presence of organic matter in the iodine solutions from Oak Ridge.

Chloride determinations on the tissue suspensions were carried out by a modification of the technique of Wilson and Ball.[17]

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TABLE II. RATIOS OF TISSUE CHLORIDE CONCENTRATION TO SERUM CHLORIDE CONCENTRATION; EACH NUMBER REPRESENTS THE AVERAGE OF FOUR DETERMINATIONS; THE PATIENTS ARE FROM THE SERIES LISTED IN TABLE I

PATIENT	THYROID	STOMACH			KIDNEY	LIVER	LUNG	ADRENAL	HEART	MUSCLE	SKIN	SPLEEN	MILLIGRAMS OF CHLORIDE PER 100 GRAMS	
		CARDIA	FUNDUS	PYLORUS										
	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	SERUM	LIVER
K. B.	0.5	0.7	0.9	0.8	0.6		0.8	0.5	0.4	0.2	1.3	0.6	370	
E. G.	0.9	0.7	0.8	0.8	0.7	0.6	0.9	1.0	0.6	0.5	0.9	0.6	375	
F. R.	0.5	0.7	0.9	0.7	0.6	0.3	0.7	0.5	0.4	0.4	0.9	0.7	330	
F. D.	0.5	0.8	0.5	0.7	1.4	0.4	0.9	0.5	0.4	0.5	0.5	0.6	330	
E. R.*	1.7	2.0	1.3	2.0	2.0	1.0	2.2	2.0	1.4		2.4	1.6		110
	80.0	16.0	9.0	15.0	2.6	1.0	3.6	2.6	2.0		2.4	1.7		
W. S.*	0.8	1.0	1.0	1.0	1.0	1.0	3.0		0.7	1.0	1.6	1.1		215
	18.0	5.7	8.0	8.0	1.4	1.0	0.4		0.6	1.0	0.9	1.2		
F. S.*		1.1	1.9	1.9	0.9	1.0	1.3	0.4	0.4	1.9		0.8		250
	21.0	12.0	9.0	11.0	1.0	1.0	1.0	1.1	0.5	0.5		1.0		

*In the last three patients, E. R., W. S. and F. S., the number on the upper line is the tissue/liver ratio for chloride, and the number on the lower line is the tissue/liver ratio for iodine in that patient. Serum chloride values were not available in these patients, and the liver was chosen for reference because chloride is not concentrated selectively in the liver.

RESULTS

Tables I and II present the findings. Table I lists the ratios of tissue I^{131} concentrations to the serum I^{131} concentrations. The ratios of tissue chloride concentrations to the serum chloride concentrations are given in Table II.

The uptake of I^{131} by the thyroid varied from 0.1 per cent to 58 per cent of the injected quantity.* The concentration of I^{131} in the stomach tissue was two or more times the serum I^{131} concentration in samples from ten of the thirteen subjects from whom stomach tissue was obtained. The ratio of stomach I^{131} concentration to serum I^{131} concentration exceeded the ratios for other tissues except the thyroid and, in one instance, the spleen. Other ratios of tissue I^{131} to serum I^{131} were usually less than unity; the lowest ratios occurred with muscle and brain. There is little evidence of concentration of iodine within the kidney substance despite the fact that the urine is the main excretory route for iodine.

Variation in the quantity of sodium iodide injected was most clearly reflected in the thyroid which took up proportionately smaller quantities of the I^{131} as the quantity of iodide injected was increased. The spleen showed a similar variation, while most of the other tissues had stable ratios of tissue I^{131} to serum I^{131} at each of the three dose levels employed. The stomach I^{131} to serum I^{131} ratios varied so much that no definite conclusion regarding the effect of varying the quantity of injected iodide could be drawn.

With increased time of survival after sodium iodide injection, the ratios of tissue I^{131} to serum I^{131} concentrations exceeded the corresponding chloride ratios most markedly with thyroid tissue. The ratios for I^{131} most nearly resembled those for chloride in those subjects who died shortly after iodide injection and who received large quantities of iodide (Subjects K. B. and E. G.). This was particularly true for the liver and kidney. The muscle to serum ratios for both chloride and I^{131} were higher than was expected; no explanation is evident.

At autopsy, the gross and microscopic anatomy of the tissues of the patients in this study did not show evidence of x-ray effect by any of the usual criteria.

DISCUSSION

High concentrations of I^{131} occur in human stomach tissue as well as the thyroid gland after the injection of labeled sodium iodide. This contrasts with the behavior of chloride, the concentration of which in gastric juice remains in osmotic equilibrium with the blood plasma, or not more than 50 per cent above the plasma chloride level.[20] The concentrating power of the gastric mucosa for chloride may be as great as for iodide, but possibly limited physiologically by the demands of osmotic equilibrium. Another halogen ion, bromide, also has been found at somewhat higher concentrations in the gastric juice than in plasma.[21]

*It is known that I^{131} from Oak Ridge, labeled "carrier-free," is not always free of measurable traces of inert iodine. As little as 1 μg of inert iodine added to the solution from Oak Ridge may possibly be a physiologically significant increment as evidenced by the difference in iodine uptake of the thyroids of patients who received approximately 1 μg of added inert iodine and those of patients who received "carrier-free" I^{131}. But this difference may be only a reflection of the longer survival times of the patients who received "carrier-free" material.

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Iodide is distributed like chloride in most of the tissues only if the tissues are sampled soon after large quantities of iodide are given. If one may judge from these moribund subjects and from data obtained in animal experimentation,[19] many days are required for administered iodide to become distributed in the body according to that distribution pattern yielded by chemical analyses.[22] Of all the tissues analyzed for iodine by chemical techniques, blood contains the least iodine with the possible exception of the brain.[4] Yet, in the present series of subjects, the blood serum generally contained more of the injected I^{131} than any of the other tissues examined with the exception of the thyroid and stomach.

Data of the kind presented here may be used to calculate the total quantity of radiation to which a patient is exposed when radioactive iodine is administered for diagnostic purposes or treatment of thyroid disorders. This has been done by Marinelli,[23] using I^{131} concentrations observed in urine, sputum, blood, and gastric juice.

Dietary iodine intake before and during tests of thyroid function with I^{131} is not always recognized as a factor of importance. In this instance no data were obtained on the state of iodine metabolism of these subjects, other than those presented in Table I. But the fact that these subjects were eating poorly and were not taking iodine-containing medicine suggests that iodine intake was more restricted in them than is apt to be the case with most hospital patients.

SUMMARY

The distribution of I^{131} in the tissues of fourteen subjects injected intravenously with varying quantities of radioactively labeled sodium iodide was measured. These subjects died from 3 to 159 hours after the administration of the iodine.

The thyroid gland contained much higher concentrations of I^{131} than did the blood serum. Stomach tissue from ten of the thirteen subjects sampled contained two or more times the concentration of I^{131} present in the blood serum. None of the other nine tissues examined showed similar ability to concentrate the I^{131}.

In tissues other than the stomach and thyroid, I^{131} was distributed like chloride only in those subjects who received large quantities of sodium iodide and who died shortly thereafter.

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SERUM CHOLESTEROL: A PROBABLE PRECURSOR OF ADRENAL CORTICAL HORMONES

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THE high concentration of cholesterol in the adrenal cortex has been well established. The sharp fall in adrenal cholesterol under conditions of increased cortical activity has been demonstrated by Long.[1] Conversion of cholesterol to cortical hormones is believed to explain the phenomenon. In their acute experiments in rats, the Yale group[1, 2] reported no significant alterations in the cholesterol levels of other tissues (brain, liver, kidney, spleen, heart, skeletal muscle, lymphoid tissue and plasma). As one reviews their data, however, one finds a rather consistent decrease of plasma cholesterol of about 20 per cent.

The capacity of the adrenal cortex, under stimulation, to deliver large amounts of steroidal hormones into the blood seems clear.[3, 4, 5] Under conditions of *intense* and *continued* stimulation, the source of large amounts of cholesterol to satisfy continuing adrenal requirements became of considerable interest. It is to be recalled that in vitro synthesis of cholesterol from acetate by surviving adrenal cortical tissue has recently been demonstrated by Chaikoff and associates.[6]

The data given below appear to indicate that the *blood* constitutes an important source of cholesterol to the adrenal cortex when the gland is forced to produce large amounts of steroidal hormones for long periods of time. Of special interest is the fact that the esterified fraction, as compared with the free cholesterol of the serum, falls selectively and severely. This suggests that the ester cholesterol rather than that free fraction is the immediate precursor of the cortical hormones, especially since 90 per cent of the cholesterol normally found in the cortex is in the ester form. No other tissue of the body is known to approach this ratio of ester to free cholesterol except (1) the blood and (2) the corpus luteum, another producer of steroidal hormones.

METHODS

All of the subjects were maintained on constant diets for metabolic balances throughout the studies. The data were obtained in the course of nine separate experiments performed on three normal men, two men with Addison's disease, one woman apparently cured of

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TABLE I

SUBJECT	AGE	SEX	WEIGHT (KG.)	DIET				CONTROL PERIOD (DAYS)	ACTH PERIOD (DAYS)	TOTAL ACTH* (ARMOUR STD. MG.)	RECOVERY PERIOD (DAYS)	REMARKS
				PRO. (GM.)	(GM.) CHO	FAT (GM.)	CAL.					
R. S. Normal	37	M	73.8	98	300	197	3,365	4	6	408	8	
R. S. Normal	37	M	69.7	98	300	197	3,365	7	8	416	6	
R. S. Normal	37	M	70.5	98	300	197	3,365	5	10†	2,000†	15	For comparison with ACTH in same subject†
G. A. Normal	22	M	74.4	103	300	173	3,170	6	6	600	5	
D. E. Normal	24	M	64.7	98	300	176	3,177	4	8	234	6	
R. D. Cushing's	20	F	62.7	95	252	92	2,216	5	9	900	10	Bilateral cortical hyperplasia at operation
V. K. "Cured" Cushing's	21	F	63.4	79	299	96	2,376	5	5	500	5	Subtotal adrenalectomy (4 mo. post-op.)
F. H. Addison's disease	43	M	65.9	103	289	158	2,990	6	5	510	0	No DCA during entire experiment
L. W. Addison's disease and diabetes	46	M	57.0	99	249	124	2,508	6	6	550	0	DCA 3 mg. per day and no insulin during entire experiment

*We are indebted to Dr. John R. Mote and The Armour Laboratories, Chicago, Ill., for the purified ACTH used in these studies.
†Cortisone 200 mg. per day. No ACTH.

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Cushing's syndrome by a 90+ per cent subtotal adrenalectomy four months previously, and another woman with active Cushing's syndrome. Data concerning the subjects and the procedure of each of the nine studies are tabulated in Table I.

Duplicate determinations of total and free cholesterol of serum were performed by the Schoenheimer-Sperry method as modified by Sperry.[7] Urinary 17-ketosteroids[8] and "11-oxysteroids"[9] were done daily.

RESULTS

Figs. 1 through 5 show the results obtained during four studies upon three normal men.

Fig. 1 represents the most detailed of these studies, blood having been drawn three times daily throughout most of the experiment for determination of serum cholesterol.

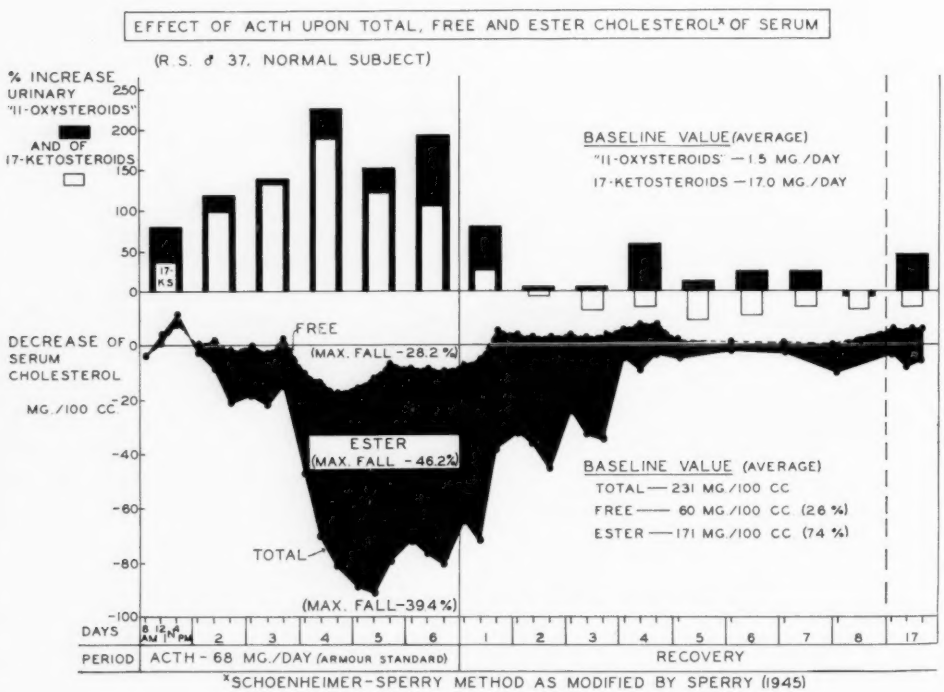

Fig. 1.

The data are charted to show the actual fall of the total and free cholesterol of serum from their average base line values. The black area between these two values represents the actual decrease of the ester fraction. For better orientation to the system used to depict these changes, the absolute values for this first experiment are shown in Fig. 2.

It is quite evident at a glance (Figs. 1 and 2) that between the third and fifth days of ACTH a great decrease of total serum cholesterol has occurred. At the point of maximal decrease this amounts to a fall of 91 mg. per 100 c.c. of serum (39.4 per cent of 231 mg.). It is clear, too, that of the 91 mg. per

100 c.c. of serum which disappeared, 79 mg. was ester cholesterol (46.2 per cent of 171 mg.) and only 12 mg. was free cholesterol. It is to be noted next that free cholesterol maintained its base line value during the first three days of administration of ACTH but that the ester fraction had begun to fall on the morning of the second day of ACTH. When the precipitous decrease of the ester fraction occurred it was followed by a decrease of the free fraction, but the latter fall was of much smaller proportions, both in absolute and in relative terms. Upon cessation of ACTH, free cholesterol bounded above its base line value in one day. On the other hand, it required six days for the ester fraction to return to its normal value.

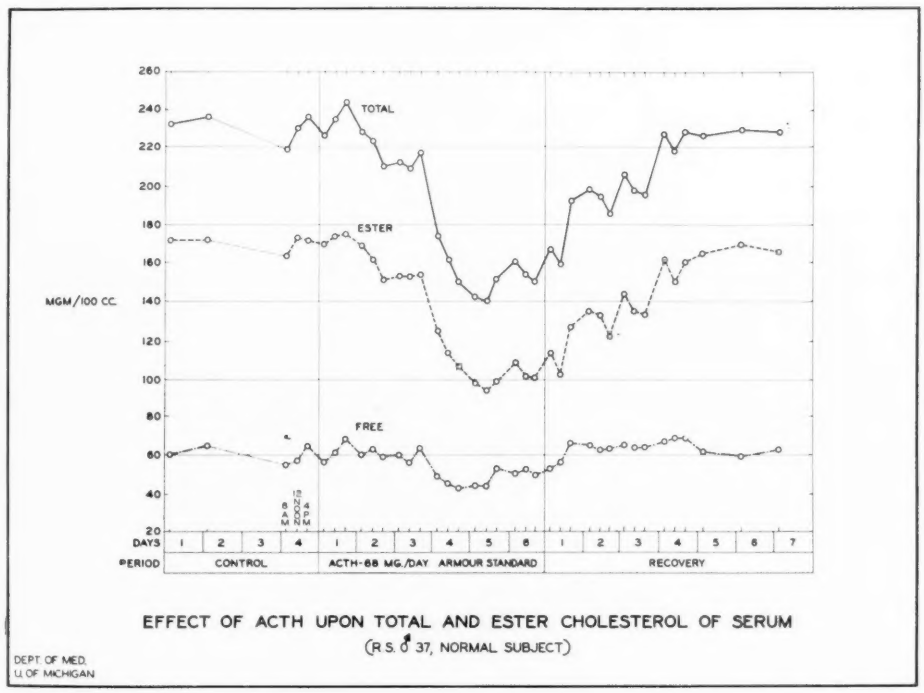

Fig. 2.

The data on urinary excretion of 17-ketosteroids and of "11-oxysteroids" are included merely to indicate that the quality and quantity of ACTH employed in these experiments were such as to produce intense and prolonged stimulation of adrenal corticosteroid production.

The other three ACTH experiments upon normal men (Figs. 3, 4, and 5), while performed with a lesser degree of detail, show the same pattern of response of free and esterified cholesterol to prolonged adrenocortical stimulation. It should be pointed out that the smaller decrease of serum cholesterol exhibited by Subject D. E. (Fig. 5) is related to the smaller doses of ACTH employed during the first three ACTH days. This is reflected, too, in the smaller increases observed initially in the excretion of 17-ketosteroids and of "11-oxy-

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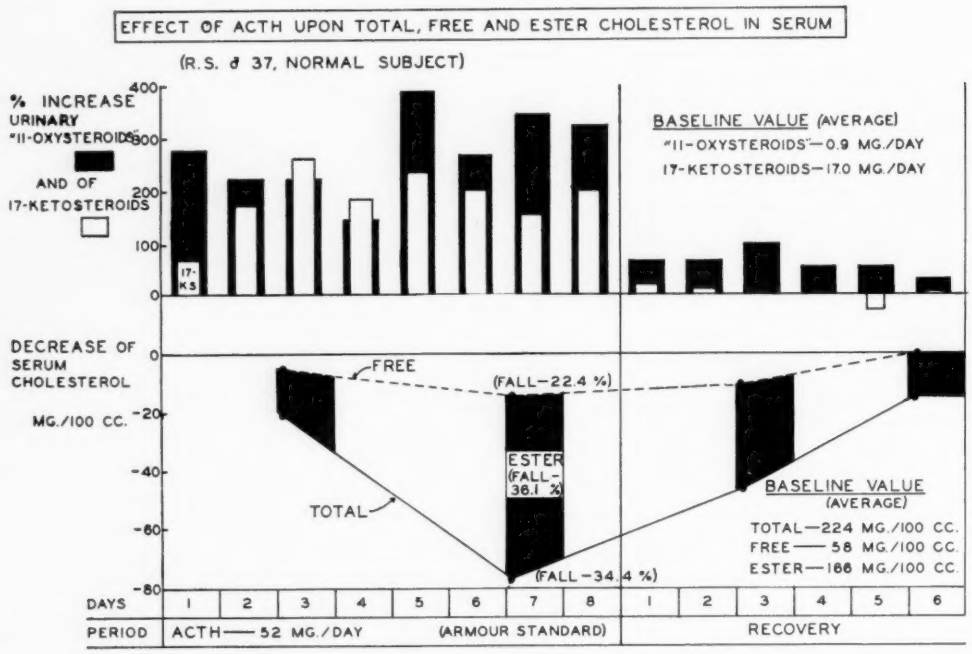

EFFECT OF ACTH UPON TOTAL, FREE AND ESTER CHOLESTEROL IN SERUM
(R.S. ♂ 37, NORMAL SUBJECT)
% INCREASE URINARY "11-OXYSTEROIDS" AND OF 17-KETOSTEROIDS
400
300
200
100
0
17-KS
BASELINE VALUE (AVERAGE)
"11-OXYSTEROIDS"—0.9 MG./DAY
17-KETOSTEROIDS—17.0 MG./DAY
DECREASE OF SERUM CHOLESTEROL MG./100 CC.
0
-20
-40
-60
-80
FREE
(FALL—22.4 %)
ESTER (FALL—36.1 %)
TOTAL
(FALL—34.4 %)
BASELINE VALUE (AVERAGE)
TOTAL—224 MG./100 CC.
FREE—58 MG./100 CC.
ESTER—166 MG./100 CC.
DAYS 1 2 3 4 5 6 7 8 1 2 3 4 5 6
PERIOD ACTH—52 MG./DAY (ARMOUR STANDARD) RECOVERY

Fig. 3.

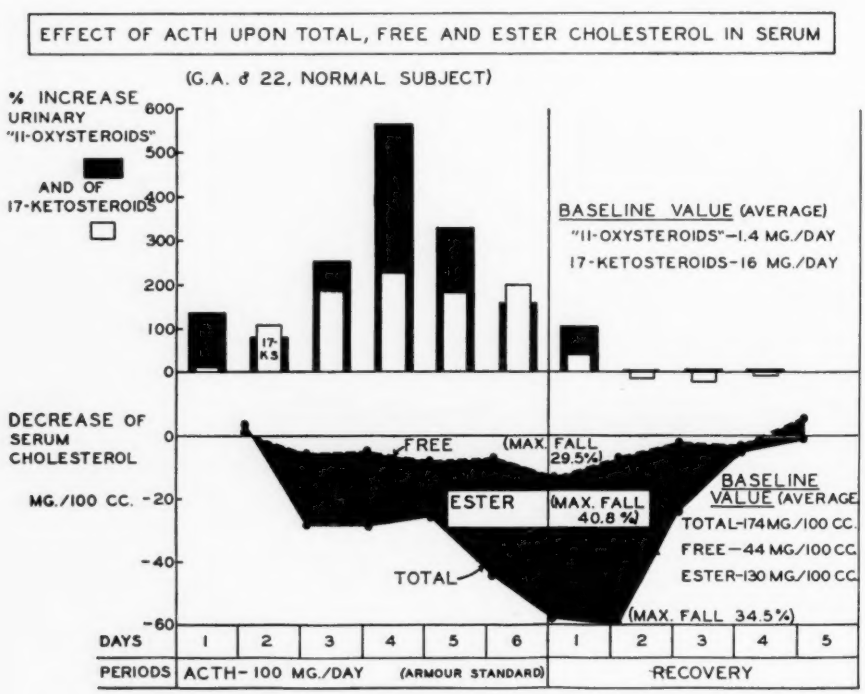

EFFECT OF ACTH UPON TOTAL, FREE AND ESTER CHOLESTEROL IN SERUM
(G.A. ♂ 22, NORMAL SUBJECT)
% INCREASE URINARY "11-OXYSTEROIDS" AND OF 17-KETOSTEROIDS
600
500
400
300
200
100
0
17-KS
BASELINE VALUE (AVERAGE)
"11-OXYSTEROIDS"—1.4 MG./DAY
17-KETOSTEROIDS—16 MG./DAY
DECREASE OF SERUM CHOLESTEROL MG./100 CC.
0
-20
-40
-60
FREE
(MAX. FALL 29.5%)
ESTER
(MAX. FALL 40.8 %)
TOTAL
(MAX. FALL 34.5%)
BASELINE VALUE (AVERAGE)
TOTAL-174 MG./100 CC.
FREE—44 MG./100 CC.
ESTER-130 MG./100 CC.
DAYS 1 2 3 4 5 6 1 2 3 4 5
PERIODS ACTH-100 MG./DAY (ARMOUR STANDARD) RECOVERY

Fig. 4.

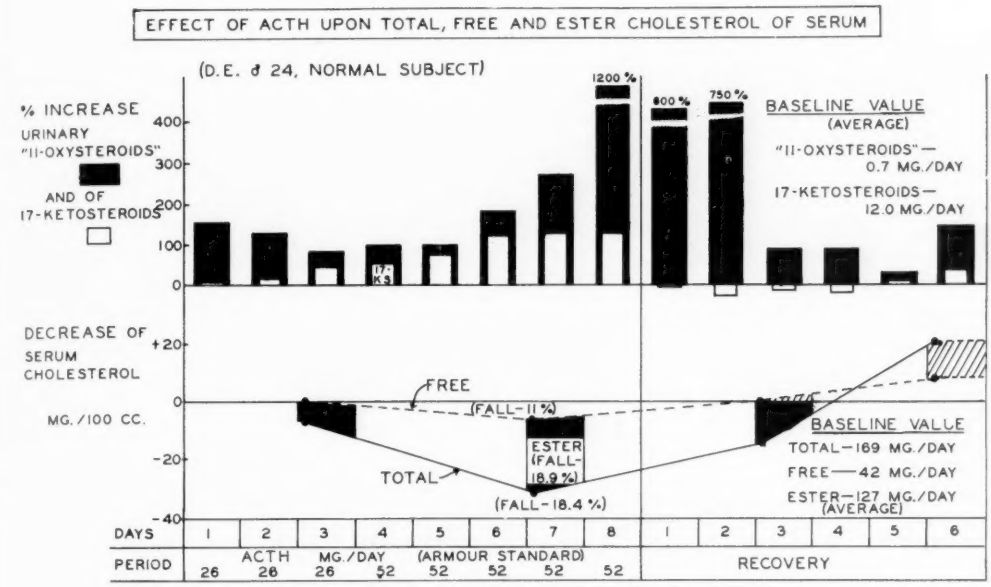

EFFECT OF ACTH UPON TOTAL, FREE AND ESTER CHOLESTEROL OF SERUM
(D.E. ♂ 24, NORMAL SUBJECT)
% INCREASE URINARY "11-OXYSTEROIDS" AND OF 17-KETOSTEROIDS
1200 %
800 %
750 %
BASELINE VALUE (AVERAGE)
"11-OXYSTEROIDS" — 0.7 MG./DAY
17-KETOSTEROIDS — 12.0 MG./DAY
DECREASE OF SERUM CHOLESTEROL MG./100 CC.
FREE
(FALL-11%)
ESTER (FALL-18.9%)
TOTAL
(FALL-18.4%)
BASELINE VALUE
TOTAL—169 MG./DAY
FREE—42 MG./DAY
ESTER—127 MG./DAY (AVERAGE)
DAYS 1 2 3 4 5 6 7 8 1 2 3 4 5 6
PERIOD 26 26 26 52 52 52 52 52 RECOVERY
ACTH MG./DAY (ARMOUR STANDARD)

Fig. 5.

EFFECT OF ACTH UPON SERUM CHOLESTEROL AND URINARY STEROIDS

(R.D. ♀ 20, CUSHING'S SYNDROME)

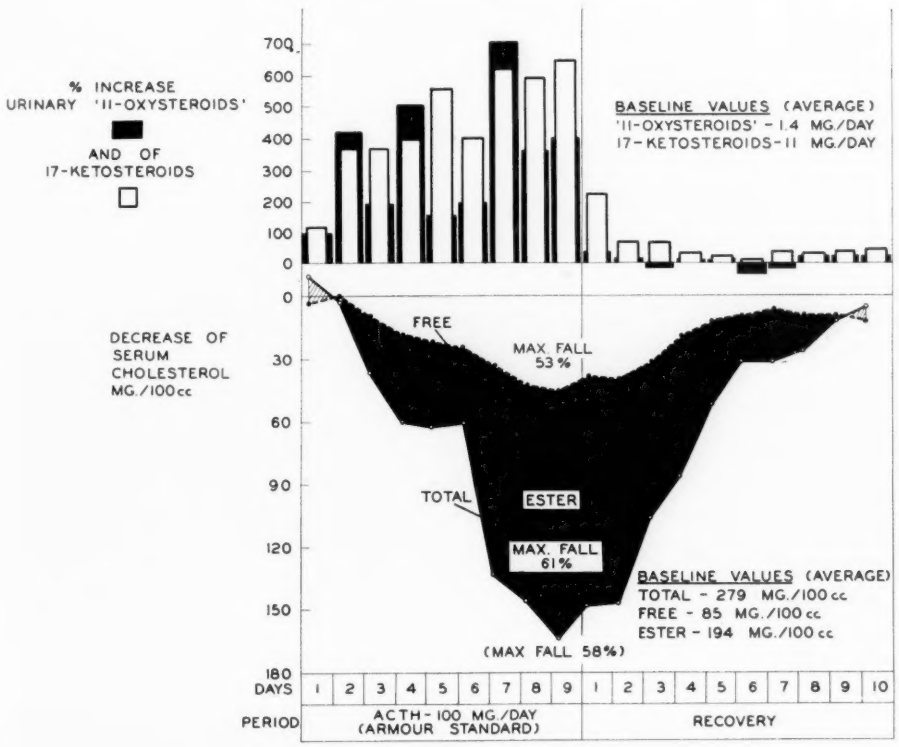

% INCREASE URINARY '11-OXYSTEROIDS' AND OF 17-KETOSTEROIDS
BASELINE VALUES (AVERAGE)
'11-OXYSTEROIDS' - 1.4 MG./DAY
17-KETOSTEROIDS - 11 MG./DAY
DECREASE OF SERUM CHOLESTEROL MG./100cc
FREE
MAX. FALL 53%
TOTAL
ESTER
MAX. FALL 61%
(MAX FALL 58%)
BASELINE VALUES (AVERAGE)
TOTAL - 279 MG./100 cc
FREE - 85 MG./100 cc
ESTER - 194 MG./100 cc
DAYS 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 10
PERIOD ACTH - 100 MG./DAY (ARMOUR STANDARD) RECOVERY

Fig. 6.

steroids." A glance at Figs. 1 and 4 (in which experiments samples for serum cholesterol were obtained daily) suggests that a much greater decrease would have been observed in Subject D. E. (Fig. 5) on the morning of the first day following cessation of ACTH. This same consideration applies with respect to the experiment on Subject R. S. (Fig. 3).

Fig. 6 represents the results of a similar experiment done upon a patient with active Cushing's syndrome whose photograph is shown in Fig. 7.

It is seen that the decrease of serum cholesterol is qualitatively the same as that observed in the normal subjects. Quantitatively, however, the response is much greater. The maximal fall of the ester fraction was 115 mg. per 100 c.c. and that of the free cholesterol was 45 mg. per 100 c.c. Note again the delay of several days before the ester fraction falls sharply.

Fig. 7.

Fig. 8 shows the results obtained in a young woman who four months previously had had a therapeutic removal of one and five-sixths of the adrenal glands with remarkable disappearance of all of the manifestations of a previously typical Cushing's syndrome. It will be observed that the base line values for the urinary steroids fell well within normal values for a 21-year-old woman. It may be significant and related to the small amount of functioning cortex remaining that 100 mg. per day of ACTH failed to evoke a greater urinary excretion of 17-ketosteroids and of "11-oxysteroids." A similar finding is observed with respect to the decrease of serum cholesterol. While qualita-

tively similar to the responses exhibited by the normal men, the degree of depression of serum cholesterol was small in relation to the dose of ACTH employed. There is a suggestion of adrenocortical fatigue on the fifth day of stimulation. The fall of serum cholesterol was less marked and less well sustained.

Fig. 9 represents a similar experiment performed upon a patient with Addison's disease. His daily dose of DCA had been discontinued at the beginning of the base line period, seven days prior to the first day of ACTH. It is noteworthy, first, that no change in the excretion of 17-ketosteroids occurred during a five-day period of large doses of ACTH; second, that during this period "11-oxysteroid" excretion fluctuated between 50 per cent above and 50 per cent below the low base line values*; and, third, that no significant changes of serum cholesterol occurred.

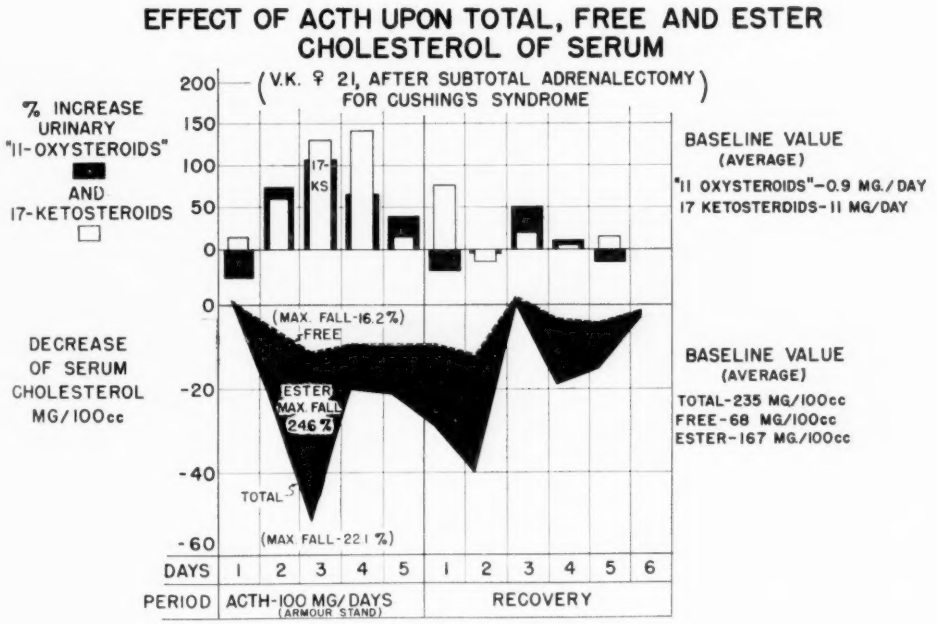


Fig. 8.

Fig. 10 reveals an interesting set of data. These were obtained from another patient with Addison's disease who in addition has diabetes mellitus. In this study DCA (3 mg. per day I. M.) was administered throughout the entire experiment. Insulin was discontinued at the beginning of the base line period. It is observed that the base line value of 17-ketosteroid excretion is at the expected value for male patients with Addison's disease. However, base line "11-oxysteroid" excretion is well within the normal range. A possible modus operandi for such dissociations of adrenal cortical function in Addison's disease will be discussed elsewhere.

*It is also worthy of mention that daily counts of circulating blood eosinophil cells showed no significant deviation from the base line values.

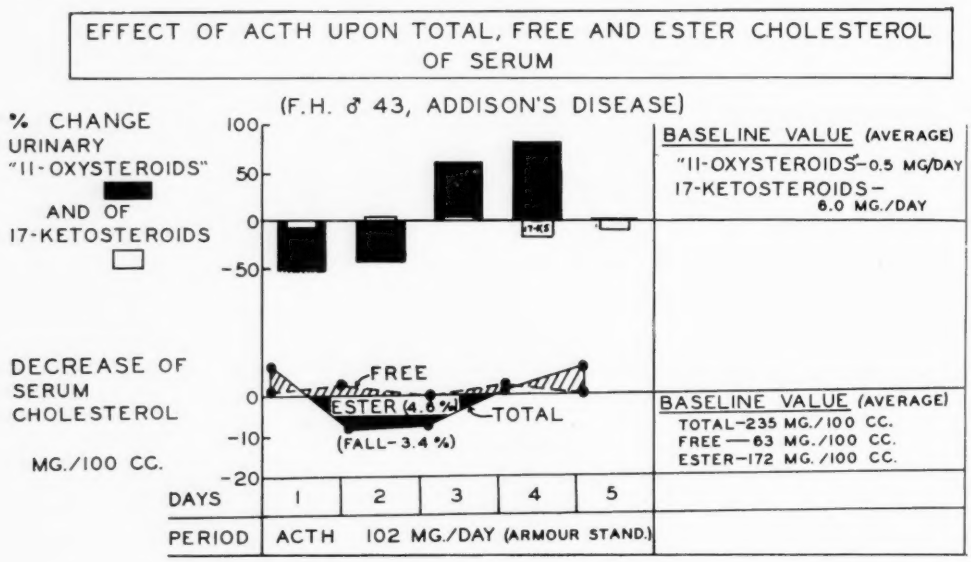


Fig. 9.

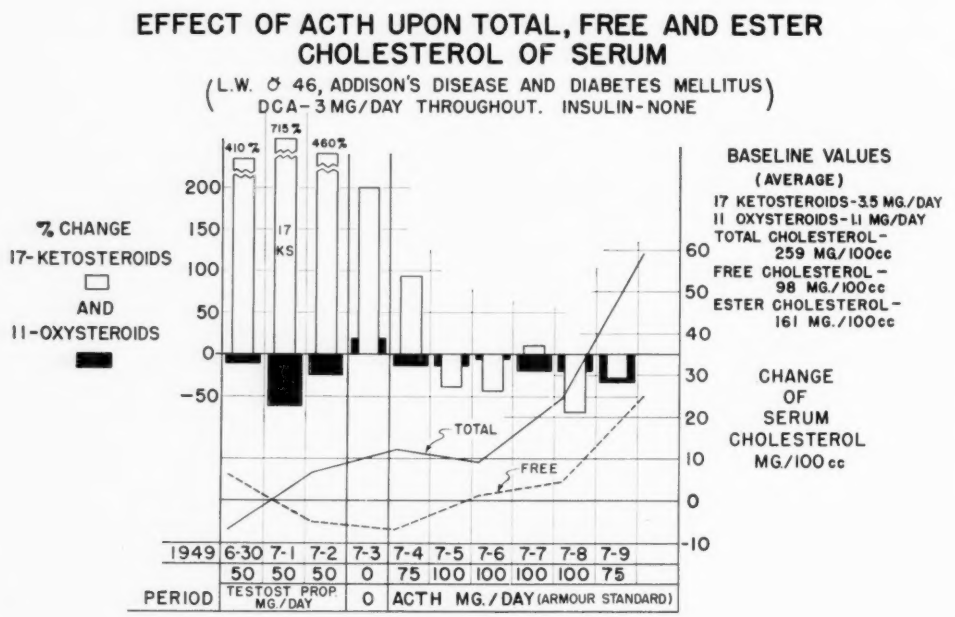
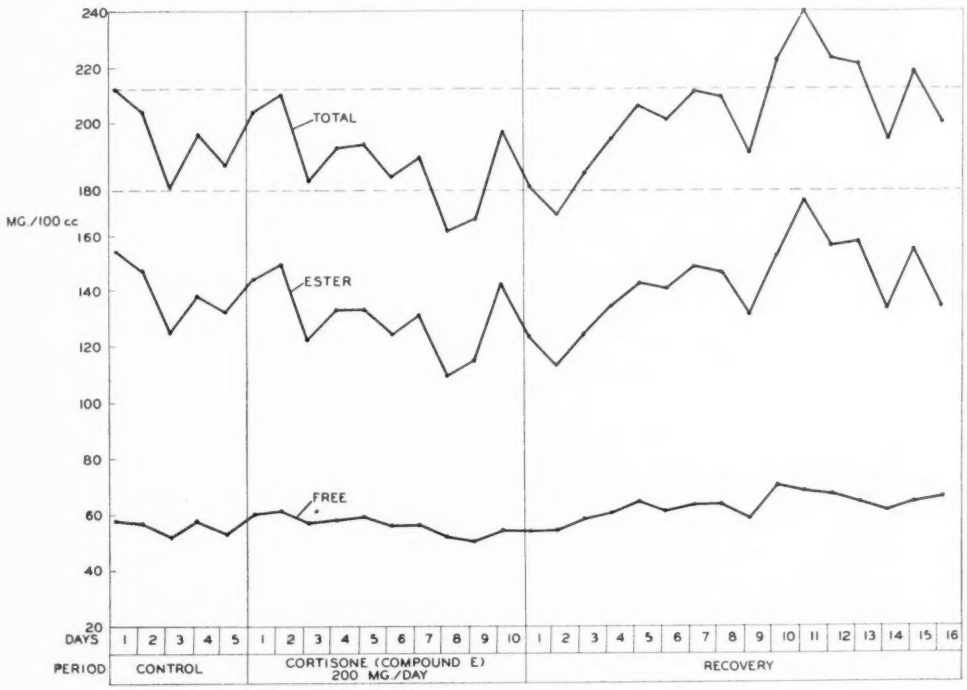


Fig. 10.

In this study a three-day period of administration of testosterone propionate preceded the ACTH period. Three points are noteworthy. First, 17-ketosteroid excretion increased greatly and persisted for forty-eight hours after the last dose of testosterone propionate. If one uses the base line value as the expected rate of excretion for the five-day period in which 17-ketosteroid ex-

cretion was excessive (three days of testosterone propionate plus the two following days), one can calculate that 43 per cent of the 150 mg. of testosterone propionate was excreted as 17-ketosteroids. If, on the other hand, one were to assume that the exogenous testosterone depressed completely endogenous production of 17-ketosteroid precursors and that all of the excreted 17-ketosteroids were derived from the testosterone propionate administered, calculation would indicate a 55 per cent conversion of this compound to excretory 17-ketosteroids. That the value will fall somewhere between 43 per cent and 55 per cent is ob-



EFFECT OF CORTISONE UPON TOTAL AND ESTER CHOLESTEROL OF SERUM
(R.S. ♂ 38, NORMAL SUBJECT)

Fig. 11.

vious. This is in close agreement with the figure of 50 per cent observed by others and indicates the normal capacity of this subject to carry out this conversion. The second point of interest is the apparent reduction of excretory "11-oxysteroids" in the testosterone period. Third, no significant change in serum cholesterol was observed during the administration of testosterone propionate.

During the ACTH there is no evidence of increased production of steroidal hormones. Excretion of 17-ketosteroids and of "11-oxysteroids" is mildly below their base line values. This may or may not reflect a continued effect of the prior administration of testosterone.

A somewhat surprising rise of both the free and ester cholesterol occurred in the latter part of the ACTH period. From the consistent fall of serum cholesterol exhibited by normal persons given ACTH and from the lack of any change observed in the prior patient with Addison's disease given ACTH it seems likely that the rise of serum cholesterol observed in this patient is related to the presence of diabetes. Insulin had been stopped eleven days before ACTH was begun. If the administered ACTH was responsible for this rise of serum cholesterol, the mechanism is obscure at present. In any case, serum cholesterol did not fall as it does in the presence of functioning adrenals.

Fig. 11 shows the results obtained when 200 mg. per day of cortisone (in place of ACTH) were administered to the same normal man who had been studied twice previously with ACTH (Figs. 1, 2, and 3). There appears to be a mild but not well-maintained depression of serum cholesterol. Whether or not it is real we are not yet prepared to say. During the entire experiment only three points fell below the lower limit of the range of base line values. It may be significant that the most intense *metabolic effects* of cortisone were observed during the five-day period immediately following *cessation* of cortisone. Yet during this period serum cholesterol, if it was changing significantly, was rising. On the other hand, the higher than base line values of the latter part of the recovery period suggest that the mild depression observed earlier *might* have been real.*

DISCUSSION

The data demonstrate that persistent stimulation of the adrenal cortices of man by administration of ACTH results in a very significant decrease of total circulating serum cholesterol, and that upon cessation of ACTH, serum cholesterol returns to its base line concentration in four to five days. On the other hand, similar treatment with ACTH of patients with Addison's disease produces no lowering of serum cholesterol. One can conclude that the adrenal cortices must be capable of greatly accelerated functional activity if the decrease of serum cholesterol is to occur. The possibility that the phenomenon might be produced by a thyrotropic contaminant in the ACTH is eliminated by the absence of any fall in serum cholesterol in the ACTH-treated patients with Addison's disease.

The fact that the fall of serum cholesterol is dependent upon an intense and continued response of the cortex to ACTH limits speculation regarding its mechanism to two major possibilities: (a) that the decrease of serum cholesterol is due to a specific activity of one or more of the cortical steroids which are produced in excess, or (b) that under conditions of forced production of large quantities of steroidal hormones, the adrenal cortices, after having depleted their own cholesterol reserves, use the blood as a further source of the compound for continued synthesis of steroidal hormones. The differences observed between the cholesterol ester fraction and the free fraction with respect to the

*Since completion of this report we have followed total and free cholesterol on two other subjects who received 200 mg. per day of cortisone for ten days. No decrease of cholesterol was observed. In fact, serum cholesterol rose mildly during the period of cortisone activity in one of them.

time that each begins to decrease, their rates of descent, their maximal falls, and their rates of return to the base line focus particular attention upon esterified cholesterol, the prime mover.

Insufficient data are available to rule out with certainty a pharmacologic effect of adrenal steroids. In a few isolated experiments blood cholesterol has been followed during administration of compound E. Sprague,[10] during studies in Addison's disease, gave the material on two occasions in doses of 20 mg. per day and 100 mg. per day respectively. No decrease of serum cholesterol was observed. More recently he[10] found no significant changes of serum cholesterol in patients with rheumatoid arthritis given 200 mg. per day of cortisone.* Perera and associates[11] noted "a slight decrease in cholesterol esters" in three hypertensive subjects given cortisone (80 mg. per day).

It may be that 400 or 500 mg. per day of cortisone will produce a change in serum cholesterol similar to that which we have observed with 50 mg. per day of ACTH. If the changes *were* due to a peripheral effect of the steroids it would imply one of two mechanisms, either decreased hepatic synthesis of cholesterol, particularly of ester cholesterol, or increased removal of cholesterol from the blood for the purpose of oxidation, fat transport, or tissue deposition. Little is known of these possibilities in relation to increased cortical function.

Favoring removal of ester cholesterol from the blood for the purpose of conversion to steroidal hormones during intense and prolonged adrenal hyperactivity are the following points.

(1) ACTH, epinephrine, or stress produce a sharp fall in total adrenal cholesterol.[1] Over 90 per cent of that which disappears from the gland is ester cholesterol.

(2) If it is accepted that the disappearance of cholesterol from the *gland* represents conversion to steroidal hormones, the predominant precursor is esterified cholesterol. That cholesterol *can* be converted to another steroidal hormone, pregnandiol, already has been demonstrated by Block.[12]

(3) The fact that adrenal cholesterol *falls* sharply with increased functional activity of the gland indicates that although the gland may be capable of synthesizing cholesterol,[6] this process is not keeping pace with the rate at which the material is being removed from the gland.

(4) The delay of several days, which we have observed, before a significant fall of circulating cholesterol occurs, suggests that under the conditions of our experiments the cortex is able to supply the needed precursors for a brief period. When the sharp fall of blood cholesterol *does occur*, it is almost entirely the esterified fraction which falls.

(5) The organic chemist finds it necessary to block the reactive hydroxyl group on carbon number 3 when he attempts to make substitutions on the cholesterol molecule. From this point of view the adrenal cortex might well select esterified cholesterol as the more immediate precursor of its steroidal hormones.

One is not justified in attempting to calculate the presumed production of corticosteroids on the basis of the total blood volume decrease of cholesterol. This is so because one does not know that the hepatic supply of cholesterol to

See footnote, page 514.

the blood is constant under these circumstances. There might well be an increasing supply to the blood as the concentration in the blood diminishes. If, however, one assumes a constant rate of supply of cholesterol to the blood under the conditions of these experiments, rough calculation gives the following interesting figures. They are listed in the order of increasing intensity of over-all metabolic responses to ACTH.

SUBJECT	OVER-ALL METABOLIC RESPONSE (+ TO ++++)	DOSE OF ACTH (MG./DAY)	CALCULATED CONVERSION OF CHOLESTEROL TO CORTICOSTEROIDS (MG./DAY)
F. H. (Addison's)	0	100	0
L. W. (Addison's)	0	100	0
V. K. (Subtotal adrenalectomy)	+	100	280
D. E. (Normal)	++	39	275
G. A. (Normal)	+++	100	426
R. S. (Normal)	++++	50	650
R. S. (Normal)	++++	68	1,138
R. D. (Cushing's)	++++	100	1,031

These are very crude calculations, probably representing only distant approximations of daily corticosteroid production. Nevertheless, the figures produced are of an order of magnitude which seems reasonable.

Finally, speculations such as those embarked upon in the preceding paragraphs necessarily lead to an appraisal of hepatic function as a possible limiting factor in the production of adrenocortical steroids. If the fall of esterified cholesterol of serum represents withdrawal from the blood of this substance for synthesis of adrenal steroids, the source of supply would be dependent predominantly upon the liver. A decreased capacity to esterify cholesterol in normal amounts, as occurs in severe hepatic insufficiency, might account for diminished adrenocortical responses to stress, especially if the stress were prolonged. This critical problem is now being studied.

SUMMARY AND CONCLUSIONS

(1) Normal people given large amounts of ACTH exhibit a sharp decrease of total serum cholesterol after several days of such treatment. The base line level is regained three to five days after cessation of ACTH. A similar but more intense effect was observed in ACTH-treated Cushing's syndrome.

(2) The esterified fraction of serum cholesterol falls sooner than the free fraction and its percentage decrease from its base line value is significantly greater than that which occurs in the free fraction. Eighty-five per cent of the decrease of total cholesterol is due to disappearance of the ester fraction.

(3) Upon cessation of ACTH, free cholesterol returns to its base line value before the esterified fraction regains its base line value. At this point in the recovery phase the deficiency of total serum cholesterol is due wholly to a diminished amount of esterified cholesterol.

(4) Patients with Addison's disease treated with ACTH in a similar way show no significant decreases in either free or esterified serum cholesterol.

(5) The data may be interpreted in one of two ways. First, that the fall of serum cholesterol is the result of the physiologic activities of one or more of the cortical steroids produced in excess when ACTH is administered or, second, that the blood constitutes a source of cholesterol for the synthesis of cortical hormones after the cortex has depleted its cholesterol reserves in the course of prolonged ACTH stimulation, the latter forcing continued production of large amounts of steroidal hormones.

(6) In the light of present knowledge and until more data are available on the specific effect of cortical steroids upon serum cholesterol, the second possibility is favored as best explaining the data.

(7) On the basis of the assumption that esterified cholesterol of serum is drawn upon during periods of acute or chronic stress as a readily available precursor with which to satisfy the increased demand for corticosteroid production, diminished hepatic function may assume the role of a limiting factor in the total response of the cortex to stimulation.

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STUDIES OF THE EFFECT OF 2,6-DIAMINOPURINE ON THE BLOOD AND BONE MARROW OF SWINE

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HITCHINGS and co-workers[1] have found that 2, 6-diaminopurine, a compound which is structurally analogous to the 2, 4-diamino derivatives of pteroylglutamic acid, is an effective inhibitor of the growth of *Lactobacillus casei*. This growth inhibition could be reversed by pteroylglutamic acid, or by either adenine or guanine at low concentrations of the diaminopurine. At the higher levels of diaminopurine, the effects were reversed by adenine but not by guanine.

Previous studies in this laboratory[2, 3] have shown that swine deficient in pteroylglutamic acid develop a macrocytic anemia, leucopenia, thrombocytopenia, and hyperplasia of the bone marrow with an increase in immature nucleated red cells which resemble the megaloblasts seen in the bone marrow of patients with pernicious anemia. Since it has been shown that there is a relationship between pteroylglutamic acid and nucleic acid metabolism[4, 5, 6, 7] and since the effects, at least in bacteria, of 2, 6-diaminopurine are reversed by pteroylglutamic acid over a narrow range of concentrations, it seemed of interest to ascertain if the administration of a "purine antagonist" such as 2,6-diaminopurine would produce changes in the blood and bone marrow of swine similar to those seen following the administration of the crude methylfolic acid antagonist.

EXPERIMENTAL PROCEDURE

A total of ten weanling Chester-White pigs, 21 to 28 days of age, were used in this study. The basal diet consisted of a special purified casein,[3] 10 per cent; sucrose, 73.8 per cent; lard, 11 per cent; and swine salt mixture,[8] 5.2 per cent. Sulfasuxidine was added to this in amounts of 2.0 per cent. The basal diet was fed in amounts of 29.9 grams (152 calories) per kilogram of body weight per pig per day. Vitamins were supplied in crystalline form by placing them in capsules and administering them orally three times a week. The quantities of crystalline vitamins were as follows (milligrams per kilogram of body weight daily): thiamine hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; choline chloride, 10.0; biotin, 0.10; 4-amino, 2-methyl naphthol hydrochloride, 0.1. In addition, all animals received Natola (Parke Davis, 55,000 units of vitamin A, 11,000 units of vitamin D per gram), 0.056 gram per kilogram of body weight per week. Full details of the experimental methods have been given elsewhere.[3, 8]

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The animals were given the diet described for two weeks in order to deplete their stores of pteroylglutamic acid. The daily oral administration of 2,6-diaminopurine monohydrate in capsules was then begun. The amounts given are described under Results.

Hematologic studies (red blood cell count, hemoglobin, volume of packed red cells, red cell indices, reticulocyte count, total leucocyte count, differential leucocyte count, and platelet count) were performed weekly on each animal throughout the entire experiment. Specimens of bone marrow were obtained by aspiration of the sternal marrow with standard 16 gauge sternal puncture needles. A small amount of marrow fluid, usually less than 0.3 ml., was withdrawn into a clean, dry syringe and thin cover glass preparations were drawn and stained with Wright's stain. Differential counts were made on 500 to 1,500 cells. Serum bilirubin was determined by the Ducci and Watson[9] modification of the method of Malloy and Evelyn.[10]

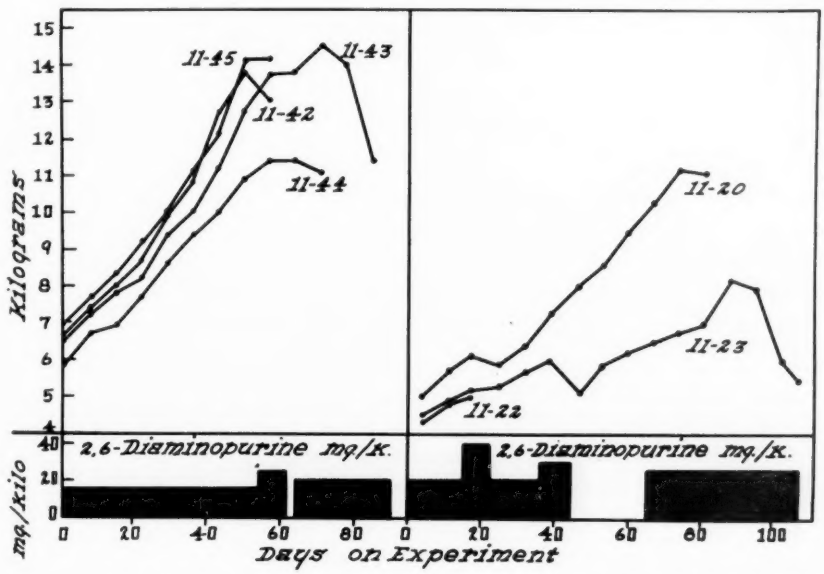

Fig. 1.—The effect of 2,6-diaminopurine administered orally on the growth of pigs.

Autopsies were performed on seven animals (Pigs 11-04, 11-20, 11-22, 11-23, 11-42, 11-44, and 11-45). The tissues which were examined routinely were heart, lungs, thyroid, spleen, liver, kidneys, stomach, intestine, tongue, skeletal muscles, and bone marrow (sternum, rib, and femur). Samples of tissue were fixed in Formol-Zenker's fluid, embedded in paraffin, and stained with hematoxylin and trichrome counterstain. The Prussian blue method was used for demonstrating iron pigment. Best's carmine stain for glycogen was used on alcohol-fixed tissue from the liver. Frozen sections of the liver were stained with Sudan IV for lipids.

For comparison in regard to growth, blood, bone marrow, and general histology, the previously reported[2] data obtained on eight pigs which were fed a similar low-protein diet were used.

RESULTS

Growth.—Three animals (Pigs 11-03, 11-04, and 11-05) were given 50 mg. of 2,6-diaminopurine per kilogram of body weight daily. One animal died on the sixth day and two on the seventh day after the compound was begun.

Three animals (Pigs 11-20, 11-22, and 11-23) were given 20 mg. per kilogram of body weight daily for fourteen days. No effect on growth was noted and the amount of antagonist was increased to 40 mg. per kilogram. After

seven days, one pig (11-22) died. The other two pigs (11-20 and 11-23) failed to gain weight (Fig. 1) and the dosage was decreased to 20 mg. per kilogram per day. On this amount growth was resumed and after fourteen days the amount of antagonist was increased to 30 milligrams. After eight days one pig (11-23) failed to gain weight and the antagonist was discontinued in both animals. After twenty days the antagonist was again administered in a dose of 25 mg. per kilogram. After twenty-one days one pig (11-20) failed to gain weight and died one week later. The other animal (11-23) continued to gain weight for twenty-three days and thereafter the weight fell off sharply; the pig died eighteen days later.

Four pigs (11-42, 11-43, 11-44, and 11-45) were started on 15 mg. per kilogram of body weight daily. Growth was unimpaired after fifty-three days and the dose was increased to 25 mg. per kilogram. Two pigs (11-42 and 11-45) failed to gain weight at this dosage level and died (Fig. 1). The other two pigs (11-43 and 11-44) failed to gain weight but survived and after seven days the antagonist was discontinued for three days and then administered in the amount of 20 mg. per kilogram. One animal (11-44) failed to gain weight and died seven days after this dosage was started. The other animal (11-43) gained weight during the first week and thereafter lost weight rapidly, dying on the twenty-seventh day after this dosage was begun.

Blood.—The results of the studies on the peripheral blood are summarized in Table I. When the drug was administered in adequate amounts, a normocytic, normochromic anemia resulted. This anemia was accompanied by a slight reticulocytosis and a slight leucopenia but there was no alteration in the number of platelets. The leucopenia was due primarily to neutropenia, but a slight absolute lymphopenia was also present in about one-half of the animals. No significant alterations in the morphology of the erythroid cells, leucocytes, or platelets were observed in the peripheral blood films. The hematologic changes in the peripheral blood of two of the pigs (11-23 and 11-43) are shown in detail in Figs. 2 and 3.

TABLE I. EFFECT OF 2, 6-DIAMINOPURINE ON THE BLOOD OF SIX PIGS

PIG	R.B.C. (MILL./C.MM.)	HGB. (GM. %)	VPRC (ML./100 ML.)	MCV (C. μ)	MCH ($\gamma\gamma$)	MCHC (%)	RETICS (%)	PLATELETS $\times$1,000 (PER C.MM.)	W.B.C. $\times$1,000 (PER C.MM.)	PMN $\times$1,000 (PER C.MM.)	MNC $\times$1,000 (PER C.MM.)
Control	6.80	12.5	37.8	56	18	33	1.5	414	15.0	7.0	8.0
11-20	5.28	11.2	31.0	59	21	36	2.4	380	11.3	1.5	9.8
11-23	3.04	5.8	17.6	58	19	33	8.8	330	7.2	5.6	1.6
11-42	5.04	10.5	31.8	63	21	33	3.7	430	12.5	5.8	6.7
11-43	2.99	7.6	22.0	73	25	34	4.9	520	8.1	3.1	5.0
11-44	4.32	8.0	26.0	60	19	31	3.3	500	10.3	4.7	5.6
11-45	4.76	9.4	30.2	63	20	31	9.2	300	9.4	1.4	8.0
Mean	4.24	8.7	26.4	63	21	33	5.4	410	9.8	3.7	6.1

VPRC, volume packed red cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PMN, polymorphonuclear cells including neutrophils, eosinophils, and basophils; MNC, mononuclear cells including lymphocytes and monocytes.

The anemia tended to develop rapidly when an effective dose of the diaminopurine was administered. As the anemia developed, a significant rise in the icterus index and serum bilirubin was noted in four out of six animals (Table II). In two of the animals (Pigs 11-23 and 11-44) (Fig. 2) the rise was considerable. There was a proportionate increase in both the prompt and the delayed fractions of the bilirubin complex (Table II).

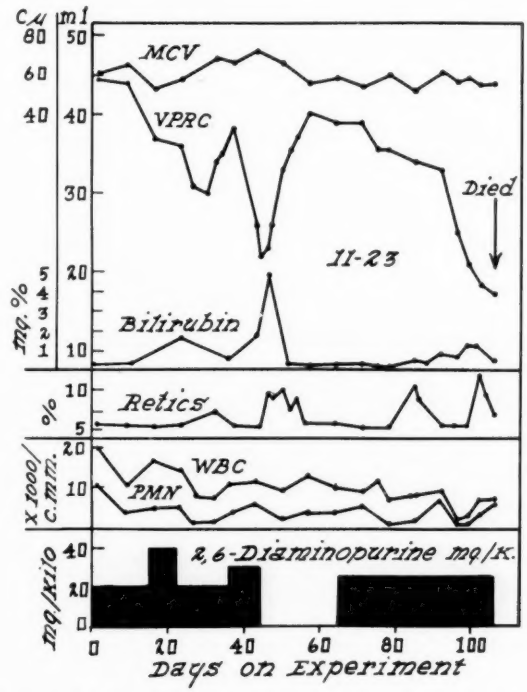

Fig. 2.—The effect of 2,6-diaminopurine on the peripheral blood of a pig. Note the spontaneous alleviation of the anemia when the administration of the drug was discontinued.

MCV refers to mean corpuscular volume; VPRC refers to volume of packed red cells; WBC refers to white blood cells; PMN refers to polymorphonuclear cells.

When the administration of the diaminopurine was discontinued, an increase in the number of reticulocytes took place and the anemia disappeared rapidly (Fig. 2). The leucocytes rose more slowly. One animal (11-43, Fig.

TABLE II. SERUM BILIRUBIN STUDIES

PIG	TOTAL (MG. %)	1 MIN. (MG. %)	T – 1 (MG. %)	ICTERUS INDEX
Control	0.15 (0.03-0.44)	0.05 (0.0-0.11)	0.10 (0.0-0.35)	3 (1-6)
11-20	0.54	0.03	0.51	10
11-23	1.34	0.45	0.89	15
11-42	0.25	0.09	0.16	3
11-43	0.80	0.22	0.58	10
11-44	4.07	1.29	2.78	50
11-45	0.19	0.06	0.13	5

T – 1, total minus 1 minute.

3) was treated with 20 mg. of pteroylglutamic acid intramuscularly daily during which time the diaminopurine was continued in a dosage of 20 mg. per kilogram per body weight. This therapy was not followed by an increase in reticulocytes; the anemia and leucopenia were not alleviated; body weight continued to decline; the serum bilirubin rose; and the animal died on the nineteenth day of pteroylglutamic acid therapy.

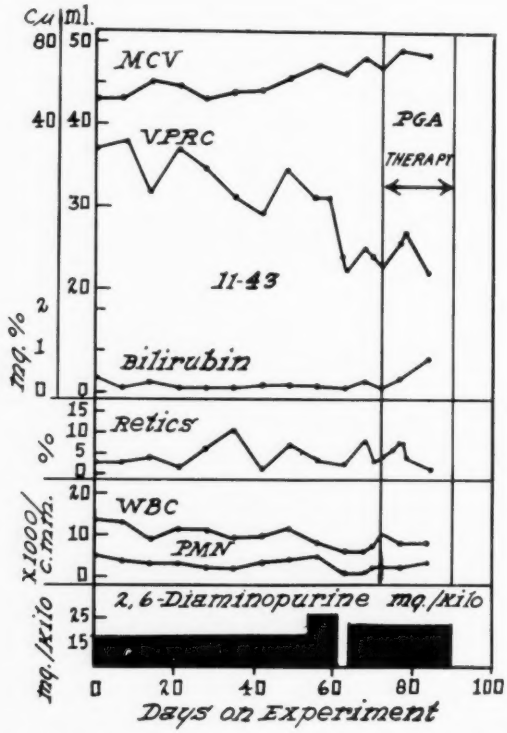

Fig. 3.—The effect of 2,6-diaminopurine on the peripheral blood of a pig. The administration of pteroylglutamic acid, 20 mg. per day intramuscularly, did not alleviate the anemia or leucopenia and did not prevent death.

MCV refers to mean corpuscular volume; VPRC refers to volume of packed red cells; WBC refers to white blood cells; PMN refers to polymorphonuclear cells.

Differential Counts on Sternal Marrow.—The results of the sternal marrow studies are summarized in Table III. The most significant abnormality was a marked increase in the leucocyte-erythroid ratio in three of the four animals studied, and a marked relative increase in the proportion of lymphocytes in all four marrows. The marrow of one animal (11-43) failed to show an increase in the leucocyte-erythroid ratio. The sternal puncture was performed in this animal eighteen days prior to death, at which time, however, a significant anemia was already present (Fig. 3). The eosinophilic myeloid series was significantly increased in the marrow of one animal (11-04) but not in the others. Cells resembling megaloblasts were not seen in any of the marrows examined.

TABLE III. BONE MARROW STUDIES

CELL TYPE	LOW-PROTEIN CONTROL		2,6-DIAMINOPURINE			
	MEAN	RANGE	11-04	11-20	11-23	11-43*
Myeloblast	0.9	0.4- 1.3	1.0	0.3	1.0	0.8
Promyelocyte	1.6	0.5- 3.8	1.0	0.6	2.5	0.4
N Myelocyte	6.8	2.6-10.0	3.2	3.0	6.5	0.8
E Myelocyte	0.8	0.5- 1.4	2.8	0.3	2.5	0.0
N Metamyelocyte	36.6	28.8-46.8	3.2	6.6	4.0	9.4
E Metamyelocyte	0.3	0.0- 1.0	19.2	0.0	0.0	0.0
PMN Neutrophil	12.4	5.3-20.6	1.2	18.0	19.0	18.4
PMN Eosinophil	0.2	0.0- 0.6	12.0	1.3	5.0	0.0
Lymphocyte	7.0	4.9- 9.3	36.8	59.4	52.0	30.0
Plasma cell	0.1	0.0- 0.4	0.4	0.0	0.5	1.2
Monocyte	0.5	0.2- 0.9	2.0	1.6	0.5	3.0
Reticulum cell	0.1	0.0- 0.2	9.6	0.3	2.0	1.6
Mitotic cell	0.4	0.0- 0.9	2.0	0.0	0.0	2.4
Megaloblast	0.0	0.0	0.0	0.0	0.0	0.0
Normoblast	32.3	21.2-49.5	5.6	8.6	4.5	32.0
Leucocyte/erythroid	2.1	1.5- 3.5	16.9	10.6	21.2	2.1

N, Neutrophilic; E, eosinophilic; PMN, polymorphonuclear.
*Eighteen days prior to death.

The changes during the course of the experiment in the relative proportions of myeloid, lymphoid, and erythroid series of cells are shown in detail in Fig. 4 in one pig (11-20). When an effective dose of the antagonist was administered there was a progressive decline in the proportion of erythroid cells, a slight decrease in myeloid cells, and a marked increase in lymphoid cells. When the administration of the antagonist was discontinued the marrow returned to normal.

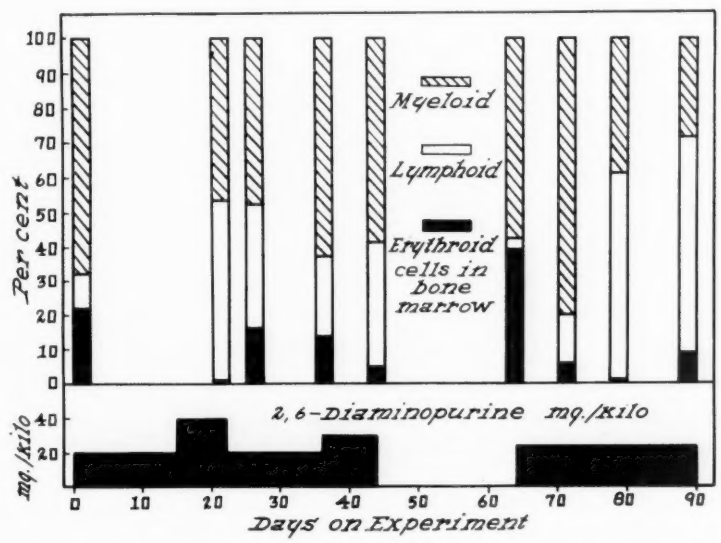

Fig. 4.—Changes in relative proportions of myeloid, lymphoid, and erythroid cells in the sternal marrow of a pig (Pig 11-20) during the administration of 2,6-diaminopurine.

Pathology.—The histologic picture in the bone marrow, with a single exception (Pig 11-44), was that of a marked reduction in the number of nucleated marrow cells. In some, the storage fat, represented by large spherical

vacuoles in the lipocytes, was apparently unaltered and the form of the cellular trabeculae was well preserved. In others, especially Pig 11-23, the depletion of fat ("serous atrophy") was extreme, some samples showing no vacuoles. In such areas the usual trabecular pattern of red marrow was lacking. Within the trabeculae of those portions of the marrow, such as the femoral marrow, which normally contain a relatively large proportion of fat, the marrow cells were reduced in number to such a degree that they were widely separated and the acidophilic matrix was clearly visible. In the sternum and ribs, where normally the cellular marrow is almost solid with cells, compact masses of cells were seen in only a few small areas and elsewhere the cells were sparse in the reticular matrix. The sinusoids were moderately enlarged in some areas and enormously dilated in others, apparently taking up the space left by the loss of marrow cells. The quantity of active hemopoietic tissues as estimated from representative areas of bone marrow ranged from 5 per cent in Pig 11-04 to 30 per cent in Pig 11-45, as compared with 40 to 100 per cent in sections from control pigs of the same age. The one exception, Pig 11-44, showed about 80 to 95 per cent of cells in sections from the sternum and ribs. Megakaryocytes semed to be less altered than the granulocytes and normoblasts. They were present in apparently normal numbers in all except Pigs 11-20 and 11-23 where they appeared to be increased in number, often appearing in clumps. In every instance they were rendered unusually prominent by the depletion of other cells of the marrow (Fig. 5).

Fatty metamorphosis of the liver was observed in samples from six of the seven pigs examined. The livers from three animals (11-04, 11-20, and 11-22) showed slight change. Stainable lipids (Sudan IV) were mainly in the form of fine droplets, concentrated in the peripheries of the lobules. In one (Pig 11-23) there was very little evidence of an increased quantity of stainable fats but the paraffin sections showed slight focal necrosis, moderately advanced granular degeneration, and slight biliary pigmentation of the parenchymal cells in the central zones. The glycogen content of the liver cells was extremely scanty in all of the livers examined.

The stomachs of all animals except Pig 11-04 presented evidence of chronic inflammation in the form of moderately dense lymphocytic infiltration of the basal zone of the mucosa and of edema of the submucosa. Marked dilatation of the capillaries associated with interstitial hemorrhage of the mucosa in its superficial zone was observed in Pigs 11-20 and 11-22. A chronic ulcer of the prepyloric area was observed in one animal (11-42). The colon of Pig 11-23 contained miliary abscesses which extended from the mucosa into the submucosa.

Skeletal muscle (gluteal and abdominal) showed various degrees of atrophy, hyaline degeneration, and necrosis, except in sections from Pigs 11-04 and 11-44. The degree of involvement varied from area to area and was of the type previously observed in protein-deficient pigs.

No significant or consistent lesions were observed in the lungs, heart, thyroid gland, tongue, kidney, or spleen.

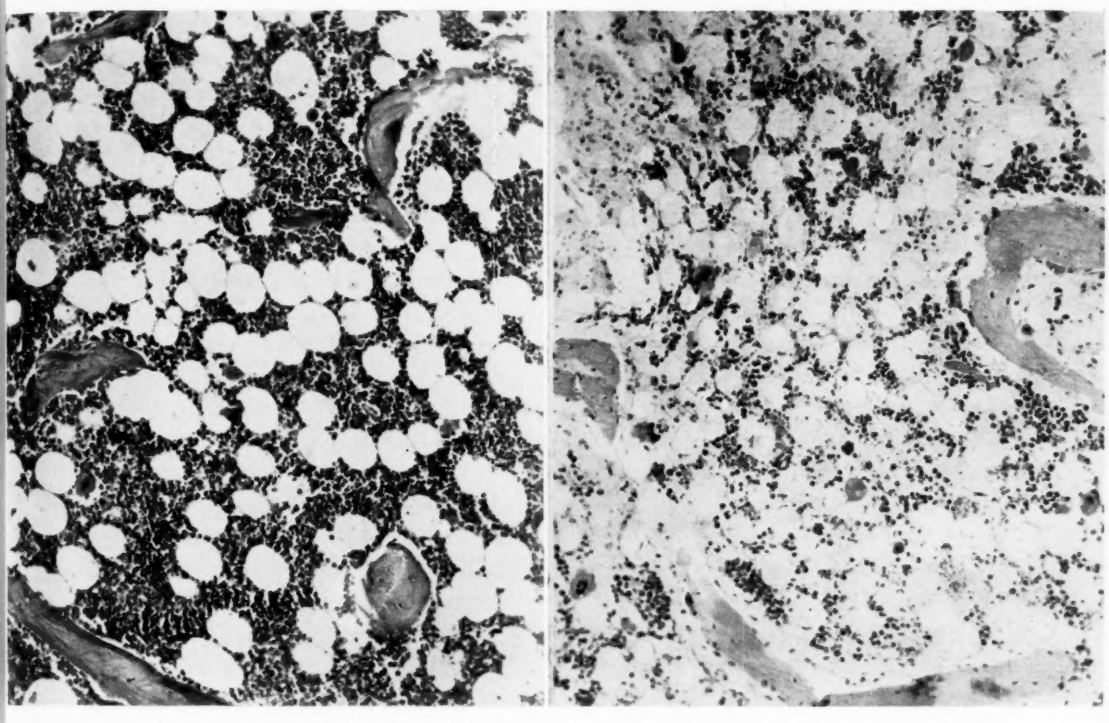

Fig. 5.—Photomicrographs of the bone marrow showing marked hypoplasia following the administration of 2,6-diaminopurine (Pig 11-23) (right). On the left, the marrow of a normal animal is shown (Pig 10-46).

DISCUSSION

The results of these experiments indicate that in growing pigs 2, 6-diaminopurine in amounts of 50 mg. per kilogram of body weight daily by mouth causes cessation of growth and death in approximately seven days. When administered for as long as fifty-three days in amounts of 15 mg. per kilogram, the antagonist fails to retard growth or impair the health of the animals. When administered in amounts of 25 to 40 mg. per kilogram the drug causes complete cessation of growth and death of the animal in several weeks' time. When the drug is given in such a dose as to cause cessation of growth, death intervenes rapidly. It would appear that there is a very narrow range between the tolerated and the toxic dose.

It is of interest that Bendich and Brown[11] have demonstrated that 2,6-diaminopurine, labeled with N^{15} in the one and three ring nitrogens and in the 2-amino group, is an effective precursor of nucleic acid guanine. They found that diaminopurine was utilized as effectively as was dietary adenine, since the amount of nucleic acid guanine derived from dietary 2,6-diaminopurine was slightly more than that derived from an equivalent amount of dietary adenine. This may help to explain the narrow range between the tolerated and the toxic dose which we have observed in swine and which Philips and Thiersch[12] have observed in mice, rats, and dogs. It may be that toxic manifestations appear only when the drug is given in such doses that all of it cannot be converted into guanine.

The hematologic manifestations which we have observed in swine following the administration of 2,6-diaminopurine are similar to those which have been observed by Philips and Thiersch[12] in mice, rats, and dogs and, in the pig at least, differ strikingly from the hematologic manifestations observed following the administration of crude methylfolic acid, a pteroylglutamic acid antagonist.[2, 3] The anemia which follows the administration of the latter is macrocytic rather than normocytic; the bone marrow is hyperplastic rather than hypoplastic; and there is an increase in early erythroid cells in the bone marrow rather than a marked depletion of erythroid elements. These differences would suggest that diaminopurine does not act as a simple pteroylglutamic acid antagonist and that the metabolic abnormality which is produced is different from that which is produced in pteroylglutamic acid-deficient swine.

The marked depletion of erythroid cells in the bone marrow suggests that the anemia is due primarily to impaired erythrogenesis. Although the fatty metamorphosis observed in the liver of six of the seven animals examined suggests that impaired liver function may have been a factor in the production of the hyperbilirubinemia, the reticulocytosis, the rapid appearance and the rapid relief of the anemia, and the accompanying disappearance of the bilirubinemia when the administration of the drug was interrupted (Fig. 2) suggest that increased blood destruction may have been an additional factor in the development of both the anemia and the hyperbilirubinemia.

SUMMARY

1. The administration of 2,6-diaminopurine to pigs in amounts of 15 mg. per kilogram of body weight per day by mouth for fifty-three days failed to retard growth or impair the health of the animals. In doses of 25 to 40 mg. the drug caused complete cessation of growth and death of the animals in about fourteen days. The administration of 50 mg. per kilogram of body weight daily resulted in death in about seven days.

2. The hematologic manifestations consisted of a normocytic, normochromic anemia, slight reticulocytosis, moderate leucopenia and hyperbilirubinemia. Differential cell counts on the bone marrows revealed a marked decrease in the proportion of erythroid cells, a slight decrease in myeloid cells, and a marked increase in the proportion of lymphoid cells. Megaloblasts were not observed.

3. Pathologically there was marked bone marrow hypoplasia, fatty infiltration of the liver, and lymphocytic infiltration and edema of the mucosa and submucosa of the stomach.

4. The hematologic manifestations differed significantly from those observed in pteroylglutamic acid-deficient swine and it is concluded that in swine the metabolic abnormality produced by 2,6-diaminopurine differs from that produced by the crude methylfolic acid antagonist.

The vitamins with the exception of pteroylglutamic acid, Synkamin, and biotin were kindly furnished by Merck and Company, Inc., Rahway, N. J., through the courtesy of Dr. A. Gibson. Pteroylglutamic acid was kindly furnished by the Lederle Laboratories, Pearl

River, N. Y., through the courtesy of Dr. T. H. Jukes. Sulfasuxidine was generously furnished by Sharp and Dohme, Inc., Philadelphia, Pa., through the courtesy of Dr. W. A. Feirer. Biotin was obtained from Hoffmann-La Roche, Inc., Nutley, N. J., through the courtesy of Dr. E. L. Severinghaus. Natola and Synkamin were supplied by Parke, Davis and Company, Detroit, Mich., through the courtesy of Dr. E. A. Sharp and Dr. E. C. Vonder Heide.

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HEMOLYTIC EFFECT OF RADIATION

Observations on Renal Bile Fistula Dogs Subjected to Total Body Radiation and on Human Blood Irradiated in Vitro

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THERE is general agreement that anemia developing in human beings and in experimental animals following exposure to either external or internal radiation may be due to impaired erythropoiesis and in some instances to hemorrhage.[1, 2] In certain instances, however, anemia has developed much more rapidly after radiation than could be attributed to lack of red cell formation, and in the absence of appreciable bleeding other explanations for the anemia have been sought. Lawrence, Dowdy, and Valentine,[1] for example, cite data of Metcalf and associates showing a sudden fall in red blood cell counts in rats starting about four days after receiving 550 r whole body radiation. The sudden decrease in circulating erythrocytes in these animals could not be adequately explained.

Although most observers have been of the opinion that mature erythrocytes are relatively resistant to x-radiation, it has nevertheless been suggested that exaggerated red cell destruction, or hemolytic processes, may contribute substantially to the anemia produced by radiation. Schwartz and associates[3, 4] have recently reviewed the available literature on the hemolytic effect of radiation and have described a number of original observations pertaining to this problem. These investigators conclude that threefold evidence supports the assumption that hemolysis may play a significant role in the production of postradiation anemia:

(1) *Histological:*

(a) Erythroid hyperplasia in human bone marrow after fatal radium poisoning.[5]

(b) Erythrophagocytosis and abundant deposits of hemosiderin in the spleen and bone marrow following acute radiation exposure[6] and ingestion of radium chloride.[7, 8]

(2) *Mathematical:* More rapid development of anemia than could be explained on the basis of complete lack of erythropoiesis after exposure to various types of radiation.

(3) *Biochemical:*

(a) Increased excretion of bilirubin in the urine by dogs with renal bile fistulas.

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(b) Increased excretion of urobilinogen in the feces by normal dogs during the first or second week after total body radiation and by patients with polycythemia vera after treatment with P^{32}.

The purpose of this report is to present the results of the following hematologic and biochemical studies on (1) renal bile fistula dogs subjected to total body radiation and (2) normal human blood radiated in vitro.

1. *Renal bile fistula dogs* were observed with respect to red blood cell counts, reticulocytes, osmotic and mechanical fragility of erythrocytes, and output of bilirubin in the urine after whole body radiation.

2. *Normal human blood* was examined for changes in osmotic and mechanical fragility of the red cells and in rates of autohemolysis and glycolysis following exposure to various doses of x-radiation in vitro.

The question of hemolytic effect of radiation on mature erythrocytes will be analyzed in the light of these observations and the need for measurements of in vivo survival of tagged radiated erythrocytes will be stressed.

MATERIALS AND METHODS

Animals. Renal bile fistulas were produced in four fully grown mongrel female dogs by anastomosis of the gall bladder to the renal pelvis according to the method of Kapsinow, Engel, and Harvey.[9] The dogs were kept in metabolism cages and were fed a standard diet consisting of hospital kitchen scraps and Purina dog chow. The diet was supplemented with water-soluble vitamin K and a multiple vitamin preparation.

Total body radiation of dogs was effected with an x-ray tube operating at 250 kilovolts and 15 milliamperes. The target-skin distance was 100 centimeters. A single dose of 150 r was delivered to one dog and a single dose of 250 r to each of three dogs at an intensity of 6.5 r per minute. Aluminum parabolic and 0.5 mm. copper filters were used. The half value layer was 2.15 mm. of copper.

Radiation of human blood in vitro was also produced with an x-ray tube operating at 250 kilovolts and 15 milliamperes. The half value layer was 0.394 mm. of copper. No filters were used. Ten milliliter samples of freshly drawn heparinized human blood from a normal donor were exposed without agitation in open sterile Petri dishes 9 cm. in diameter. The average thickness of the layer of blood in the dishes was 0.16 centimeter. The target distance and intensity of radiation were varied as explained in connection with the individual experiments. Untreated control samples were handled in exactly the same manner except that they were not exposed to radiation.

Chemical determinations. The oxidation method of Evelyn and Malloy[10] for measurement of *urinary bilirubin* was chosen in preference to other methods[11, 12, 13] on the basis of preliminary trials. *Blood sugar* was determined by the method of Nelson.[14]

Red and white blood cells were counted in duplicate using standardized hemocytometers and diluting pipettes. Blood smears were made on cover slips and stained with Wright's stain. *Reticulocyte counts* were made on blood stained with a saline suspension of brilliant cresyl blue and counterstained with Wright's stain. In determining the per cent reticulocytes, 1,000 cells were examined.

Blood for the afore-mentioned counts and smears was obtained by incision of the dog's ear vein with a Bard-Parker blade.

Determinations of *fragility of erythrocytes* were made with heparinized venous blood. Osmotic fragility was measured by the method of Shen, Ham, and Fleming,[15] and mechanical fragility was measured according to the method of Shen, Castle, and Fleming.[16] The rotator used for subjecting red corpuscles to the trauma of rolling glass beads has been described previously.[17] The distance from the center of the flask to the center of the rotating wheel employed in this procedure is 8.0 centimeters. Duplicate samples of 0.5 ml. of heparinized

dog blood, with hematocrit adjusted to 35 per cent, were rotated for ninety minutes at 28 revolutions per minute in 50 ml. Erlenmeyer flasks containing ten glass beads of uniform size and shape. The samples of human blood were treated in the same manner except that the hematocrit was not adjusted and the speed of rotation was 61 revolutions per minute. Determinations of both osmotic and mechanical fragility were carried out at room temperatures of 22° to 28° C.

RESULTS

Whole Body Radiation of Renal Bile Fistula Dogs.—Dog 48-159 was subjected to 150 r and Dogs 48-16, 48-168, and 48-149 were each given 250 r total body radiation. All of the dogs were fully grown mongrels weighing between 25 and 35 pounds prior to exposure. Dog 48-159 did not appear ill at any time following radiation, while Dogs 48-16 and 48-168 vomited infrequently and ate somewhat less food but otherwise showed no outward effects after exposure. Dog 48-149 ate very little food after radiation and died on the eighteenth day with extensive hemorrhage into nearly all organs. The marrow of the humerus, spine, and ribs was largely replaced by blood. The renal bile fistula was found to be intact and free of infection.

During the first week after radiation there was a substantial decrease in total white cell count in each of the four dogs, as shown in Fig. 1. The fall in red cell count in Dog 48-159 was to some extent attributable to the withdrawal of blood samples amounting to approximately 150 ml. during the first week. The slight decline in red cells during the second and third weeks in Dog 48-168 could also be ascribed in part to this cause, although smaller samples were taken. Withdrawal of blood was minimized in the other two dogs by sampling only a few drops at a time from an ear vein. The red cell count did not fall significantly in Dog 48-16. Hemorrhage was considered an important factor in causing the progressive anemia in Dog 48-149.

In the three surviving dogs the excretion of bilirubin through the renal bile fistulas into the urine increased markedly during the first week after radiation and there were lesser increases during the second and third weeks in two of the dogs (Fig. 1). In Dog 48-149, on the other hand, the rise in bilirubin excretion occurred from the ninth to the fourteenth day. When this dog died on the eighteenth day, extensive hemorrhages were found as previously stated. Bilirubin derived from the extravasated blood could, therefore, have contributed materially to the quantities measured in the urine of this animal.

Neither the osmotic nor the mechanical fragility of the erythrocytes was significantly altered in Dogs 48-159 and 48-168. Determinations were made every three or four days during the first three weeks after radiation. There was considerable fluctuation in the results of the mechanical fragility tests, but no more than customarily observed in normal dogs in this laboratory. These results are not charted.

In none of the four dogs were there significant changes in reticulocyte count following radiation. The highest reticulocyte count recorded for the group was 2.2 per cent in Dog 48-168, thirty days after exposure. Neither

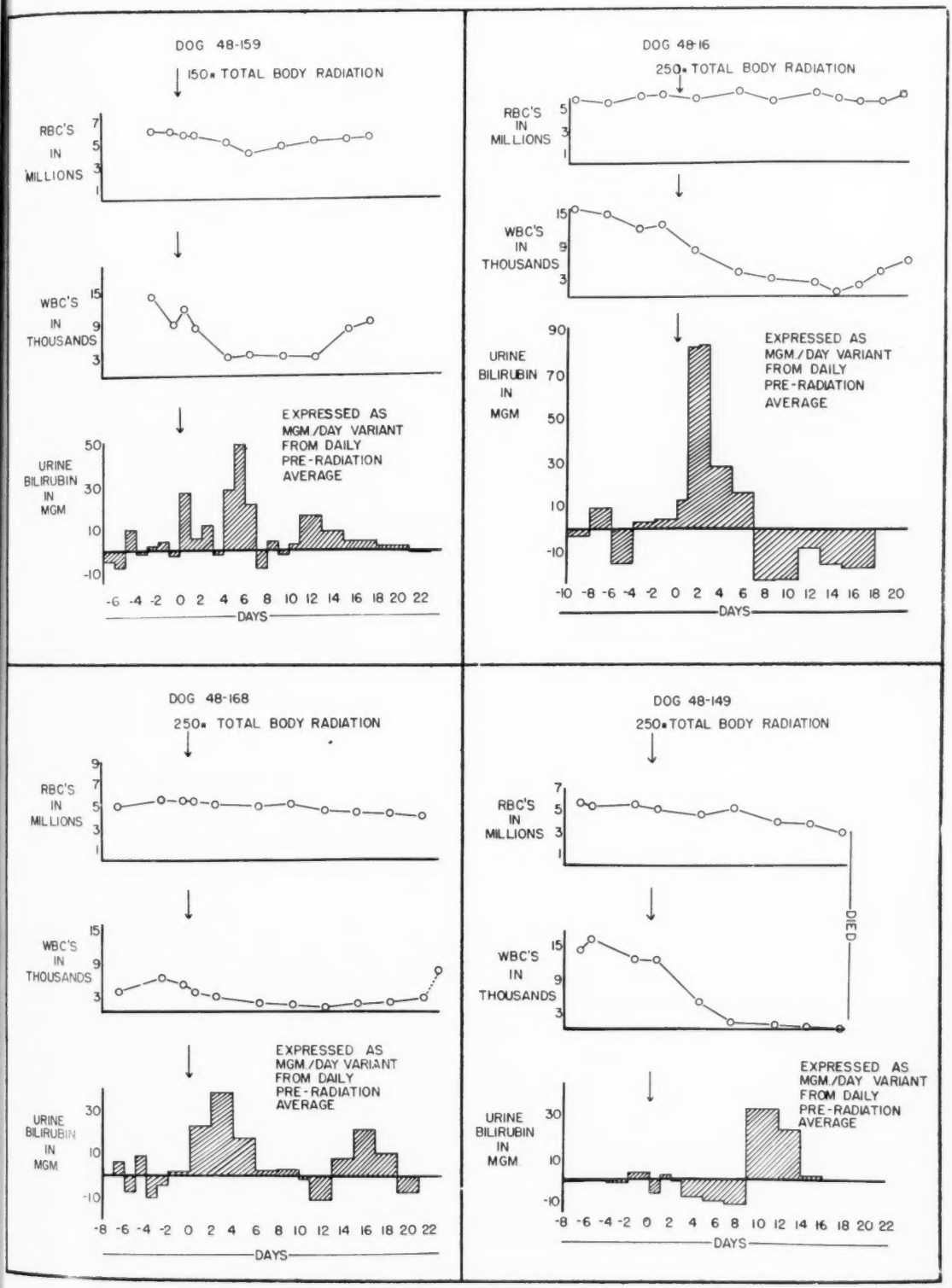

Fig. 1.—Fluctuations in red and white blood cell counts and in excretion of bilirubin in the urine of renal bile fistula dogs before and after total body radiation. The preradiation mean for bilirubin excretion in Dogs 48-159, 48-16, 48-168, and 48-149 was 26.4 mg., 41.9 mg., 39.6 mg., and 15.4 mg. per day respectively.

erythrophagocytosis nor morphologic changes in the red corpuscles could be detected in blood smears. The only dog showing marked thrombocytopenia was Dog 48-149 which succumbed following extensive hemorrhage.

Determinations of Osmotic and Mechanical Fragility and Autohemolysis of Human Erythrocytes Following in Vitro Radiation.—Heparinized blood from a normal human donor was in each of two experiments divided among four Petri dishes, three of which were radiated as previously described, and the fourth aliquot was used as a control. Human blood was used in preference to dog blood in this study because dog erythrocytes were known to give much more irregular results in tests of mechanical fragility. Measurements of osmotic and mechanical fragility of the red cells were made immediately after radiation and again after the radiated and control samples had been incubated at 37° C. for twenty-four hours. In the first experiment doses of 250, 500, and 1,000 r were delivered with an intensity of 250 r per minute and a target distance of 41.9 centimeters. In the second experiment doses of 5,000, 10,000, and 20,000 r were delivered with an intensity of 2,250 r per minute and a target distance of 15.2 centimeters.

The osmotic fragility of the red cells in the six radiated aliquots did not differ significantly from that of the two nonradiated portions when tested without incubation, and the results for the entire group of fresh samples were within the range considered normal in this laboratory.[18] Of the aliquots tested after incubation, only the portion previously subjected to a radiation dosage of 20,000 r showed slightly greater osmotic fragility than did the untreated samples. Further details of these essentially negative results are omitted for the sake of brevity.

The results of the measurements of mechanical fragility of radiated erythrocytes before and after incubation are summarized in Table I. The hematocrit was 42 per cent in the fresh samples and 49 per cent in the incubated samples and was not adjusted to 35 per cent, as is customary. The speed of rotation, moreover, was set at 61 revolutions per minute instead of the usual 28 revolutions per minute. The figures recorded agree well with those obtained with normal blood of similar hematocrit rotated under similar conditions. Radiation, in the dosage used, appeared to have no effect on the susceptibility of the cells to lysis by the trauma of rolling glass beads.

The small amounts of hemoglobin liberated as a result of autohemolysis in the samples incubated for twenty-four hours were measured and found to be no greater in the radiated portions than in the untreated aliquots.

TABLE I. MECHANICAL FRAGILITY OF RADIATED HUMAN ERYTHROCYTES BEFORE AND AFTER INCUBATION AT 37° C.

	NONRADIATED CONTROL SAMPLES		SAMPLES GIVEN STATED DOSAGE OF RADIATION IN ROENTGEN UNITS					
	NO. 1	NO. 2	250	500	1,000	5,000	10,000	20,000
Per cent hemolysis of fresh blood	2.9 14.2	3.9	5.5	5.7	1.2	3.9	3.7	3.6
Per cent hemolysis of incubated blood		17.9	12.9	13.9	13.9	17.9	16.0	12.2

Measurements of Rates of Glycolysis in Human Blood Following in Vitro Radiation.—Since glycolysis appears to be essential for maintaining the viability of erythrocytes,[19] it was deemed worth while to measure the rates of disappearance of sugar from samples of radiated blood. Aliquots of freshly drawn defibrinated human blood from a normal donor were placed in sterile Petri dishes and radiated as previously described with doses of 10, 100, 1,000, and 10,000 roentgens. The dose of 10 r was delivered with a milliamperage of 5.0, intensity of 7.38 r per minute, and target distance of 142.8 centimeters. The doses of 100, 1,000, and 10,000 r were delivered with a milliamperage of 15.0, intensity of 600 r per minute, and target distance of 26.0 centimeters. The sugar content of the radiated samples and of a nonradiated control sample were determined immediately after radiation and again after incubation at 37° C. for three hours. The glycolytic rates, expressed as milligrams loss of sugar per 100 ml. of packed erythrocytes per hour, were 28, 29, 32, and 30 mg. respectively in the radiated samples and 28 mg. in the untreated control samples. The rates of disappearance of sugar in the radiated aliquots were not significantly different from those in numerous untreated samples of normal human blood tested in this laboratory.[20]

DISCUSSION

The increased excretion of bilirubin in the urine of the four renal bile fistula dogs described in these experiments and in the studies of Schwartz and associates[3, 4] is not easily explained. The same statement may be made with regard to the increased excretion of urobilinogen in the feces of patients with polycythemia vera after treatment with P^{32} and in the feces of normal dogs exposed to total body radiation.[3, 4] Several possible sources of the bile pigment must be considered.

1. Absorption of bilirubin from areas of hemorrhage could account for much of the rise in bilirubin excretion in Dog 48-149 from the ninth to the fourteenth postradiation day. In the other animals reported, however, and in the polycythemic patients[3] there were no outward manifestations of hemorrhage and the rise in pigment excretion was observed prior to the period when hemorrhage was most apt to occur as a result of radiation. The three dogs that survived radiation were not subjected to histologic examination, and such examinations have not been made systematically in the course of the reported studies dealing with the question of hemolytic effect of radiation. It is possible, but to us it seems unlikely, that total body exposure of dogs to 150 or 250 r could produce microscopically detectable hemorrhage sufficient to cause two- or threefold increases in bilirubin excretion. This is a matter worthy of more intensive investigation.

2. Destruction of circulating erythrocytes might have been exaggerated after exposure of the animals despite the fact that the red cells of the exposed dogs appeared normal in blood smears and had normal susceptibility to both osmotic and mechanical lysis. In general there is good correlation between the results of mechanical fragility tests and the ability of erythrocytes to survive in vivo.[16, 21] It is possible, however, that cells exposed to x-radiation

may constitute an exception to this general rule, especially if they are rendered more susceptible to phagocytosis, or if the phagocytic cells of the body are induced by irradiation to engulf erythrocytes more actively. Sludging of the blood, which might be expected after total body exposure, causes red cells to be ingested and destroyed in phagocytic cells of liver and spleen, according to Knisely and co-workers.[22] In our experiments, erythrophagocytosis was not observed in blood smears from the radiated dogs; the tissues were not examined to detect sludging or extravascular phagocytosis. The histologic findings in the one dog that succumbed were distorted by hemorrhage.

The absence of anemia in Dog 48-16 and the rather unimpressive anemia in the other three dogs (due partly to hemorrhage in one dog and to withdrawal of large samples of blood from the other two dogs) does not exclude the possibility of a hemolytic process. A two- or threefold increase above the normal rate of red cell destruction (and corresponding increase in bilirubin excretion) in the dogs would not be expected to cause easily measurable decrements in red cell count (or hemoglobin) unless the hemolytic process persisted for periods longer than the observed periods of increased bilirubin excretion. Even if erythropoiesis were impaired to the extent that no new red cells were being delivered from the marrow, a mild transient hemolytic process would be difficult to detect solely on the basis of declining red cell count or hemoglobin.

3. Bilirubin was quite likely derived in some measure from destruction of partly hemoglobinized normoblasts in the marrow, since these cells are known to be readily destroyed by radiation.[6, 23] Lack of reticulocyte response in the presence of mild anemia would be expected if normoblasts were destroyed more readily than mature erythrocytes.

4. Bilirubin might have been derived from heme procursors that were not successfully incorporated into erythroid cells in the marrow. London, Shemin, West, and Rittenberg[24] have shown that in pernicious anemia a large part of the stercobilin in the feces is derived from a source other than the hemoglobin of circulating erythrocytes. These authors suggest that in patients with this disease the bile pigment may be formed from one or more of the following sources: (a) hemoglobin liberated from red cells destroyed in the marrow or soon after reaching the peripheral blood; (b) porphyrins not used in production of hemoglobin; (c) direct synthesis of bile pigment by pathways not involving degradation of a porphyrin ring. Whether or not such a diversion of pigment might occur as a result of radiation remains to be established.

5. Breakdown of myoglobin might conceivably cause an increase in bilirubin excretion, but there is no evidence that this occurs after radiation.

The observations on red cells radiated in vitro confirm the opinions of others that enormous doses are required to produce demonstrable changes. The experiments of Ting and Zirkle[25] are especially worthy of mention. These authors found that when samples of human or ox blood were exposed to 33,000 r, hemolysis could be detected within fifteen to twenty hours and the cells swelled because of increased permeability to salts.

It is not surprising that in our experience doses of 10,000 to 20,000 r failed to alter the morphology, osmotic and mechanical fragility, and glycolytic activity of freshly drawn human red cells. When the cells were incubated at 37° C. for twenty-four hours after exposure, only those treated with 20,000 r showed slightly greater increase in osmotic fragility than did other incubated corpuscles. The mechanical fragility of the cells incubated after exposure to 20,000 r did not differ significantly from that of untreated control cells or of cells given smaller doses of radiation. The tests of incubated cells were carried out because it had been shown that red cells from patients with hereditary spherocytosis (congenital hemolytic anemia) show much greater increases in osmotic and mechanical fragility after sterile incubation than do normal red corpuscles.[18, 26]

Autohemolysis of a sort which probably does not involve immunologic factors takes place much more readily in incubated blood from patients with hereditary spherocytosis than in blood from patients with a variety of other hematologic disorders or from normal donors.[18, 27, 28] It is therefore of interest that erythrocytes subjected to x-radiation in doses as large as 20,000 r failed to undergo autohemolysis more rapidly than did untreated cells or cells exposed to smaller doses.

Incubation for periods longer than twenty-four hours after radiation might make more subtle changes apparent, but it should also be kept in mind that in vitro experiments with erythrocytes may be difficult to control if continued for long periods of time. Ting and Zirkle[25] found that osmotic fragility and swelling of red cells increased steadily when the cells were kept at 5° C. for periods up to twenty-four hours after exposure to 33,000 roentgens. It therefore appears that important changes may occur slowly even at refrigerator temperatures in erythrocytes previously radiated with very large doses.

The alterations produced in red corpuscles in vitro by enormous doses of radiation are of considerable theoretical interest but they may be unrelated to the effects, if any, which relatively small doses have on mature erythrocytes in vivo. Since the evidence for a true hemolytic effect of radiation on erythrocytes in vivo is largely indirect, the occurrence of this phenomenon cannot yet be considered established. It is apparent that experimental approaches other than those thus far used should be explored in an effort to elucidate this problem. Schwartz and co-workers[3] have suggested that studies employing irradiated erythrocytes with isotopic carbon or nitrogen in the hemoglobin molecule may throw considerable light on this issue. The fate of erythrocytes transfused after either in vivo or in vitro radiation might also be determined by the technique of differential agglutination of red cells with appropriate antisera. Methods for selective agglutination of dog erythrocytes are under investigation in our laboratory at the present time and may prove useful in measuring the in vivo survival of dog red corpuscles that have been irradiated under a variety of conditions. The possibility that the effect of irradiation may not be directly upon the red cells, but upon the phagocytic cells of the body, also deserves further study.

SUMMARY

1. Four renal bile fistula dogs were exposed to total body radiation in single doses of 150 r (one dog) and 250 r (three dogs). The excretion of bilirubin in the urine by each of the four dogs increased significantly during the first or second week following exposure. Possible sources of the increments in bilirubin output are discussed and it is considered that accelerated destruction of mature erythrocytes could be neither established nor disproved by these experiments. It is considered likely that some bilirubin is derived from destruction of partly hemoglobinized normoblasts in the marrow. It is also suggested that somewhat more than the normal portion of the total bilirubin excretion might stem from hemoglobin precursors if such molecules should be less successfully incorporated into erythroid cells after radiation.

The morphology of the red cells did not appear to be altered in any of the radiated dogs, and serial measurements of osmotic and mechanical fragility of the erythrocytes revealed no significant alterations after radiation in the two dogs tested. Reticulocytosis and erythrophagocytosis could not be detected in smears of venous blood prepared after exposure.

2. Ten milliliter aliquots of heparinized blood from a normal human donor were exposed in vitro to x-radiation in doses ranging from 10 to 20,000 roentgens. The osmotic and mechanical fragility of the red cells was normal when tested immediately after radiation and again after incubation at 37° C. for twenty-four hours following exposure, except in the sample treated with 20,000 r which showed slightly greater than normal increase in osmotic fragility after incubation. Rates of autohemolysis and glycolysis during incubation in vitro were neither accelerated nor decelerated by radiation of the blood prior to incubation.

3. It is emphasized that any alterations produced in erythrocytes in vitro by very large doses of x-radiation may be unrelated to the effects, if any, which small doses have on mature red cells in vivo. It is concluded that new experimental approaches must be utilized to determine whether radiation has a truly hemolytic effect on mature red cells in vivo.

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THE RELATIVE SUSCEPTIBILITY OF SAMPLES OF HUMAN ERYTHROCYTES TO AGGLUTINATION BY INFLUENZA VIRUS

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ERYTHROCYTES from several species, including man and chickens, are agglutinated by the influenza virus.[1-5] This agglutination may be inhibited not only by agents that act on the virus itself, such as specific influenza antiserum[1, 2] and other substances,[6-11] but also by certain agents that alter the erythrocytes. Thus Hirst[12] showed that exposure to influenza virus with subsequent elution rendered the cells refractory to agglutination on the addition of fresh virus. More recently it was shown by Green and Woolley[6] that cells treated with pectin and then washed were resistant to agglutination by influenza virus. Burnet, McCrea, and Stone[13] demonstrated that a similar effect on human red cells is obtained by exposure to *Clostridium welchii* toxin Type A or to filtrates from cultures of certain strains of *Vibrio cholerae*. Also an extract of Type 5 pneumococci that had esterase activity was shown by Svec and Forster[14] to render chicken red cells refractory to agglutination by PR8 influenza virus.

That erythrocytes from apparently normal individuals within a species may differ in their agglutinability was shown by Clark and Nagler.[3] Working with influenza B, they found that cells from six of thirteen horses and one of two donkeys were refractory, while the cells from the other animals in these groups were readily agglutinated. Similarly, Miller and Stanley,[15] employing cells from a group of six hens for influenza A titrations, found a maximum variation of 36 per cent in the apparent titers obtained. Another type of variation among individuals of a species was demonstrated by Stuart-Harris[16] and by Anderson, Burnet, and Stone[17] in a related reaction. They found that chicken cells vary in their susceptibility to a nonspecific factor present in normal human sera that inhibits hemagglutination with the influenza virus.

In view of these findings it seemed desirable to determine the degree of uniformity of agglutination of human red cells in health and disease. It was also considered that such a study would be of value in determining the reliability of influenza hemagglutination inhibition tests in which human red blood cells are used for the titration of influenza antibodies. Erythrocytes that were derived chiefly from an unselected series of patients in a general hospital were tested to determine the relative concentration of virus required to cause agglutination. In addition, samples were similarly tested from presumably normal individuals. Most of the samples from the patients gave results similar to those in the control group. Decreased agglutinability was shown by individual specimens from two patients with pulmonary disease.

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MATERIALS AND METHOD

Virus.—The PR8 strain of influenza A virus, kindly furnished by Dr. Thomas Francis, Jr., was used throughout. This was propagated by inoculation into the allantoic cavity of 12-day-old chick embryos. The allantoic fluid was harvested two days later and partially purified virus suspensions were prepared according to the method of Francis and Salk.[18] The hemagglutination titers of these suspensions varied from approximately 1:4,000 to 1:10,000 when titrated with human red cells by the technique described below.

Cells.—Human red cells were obtained by transferring blood from a finger prick to 10 ml. of saline containing 2 per cent sodium citrate. The cells were washed three times in saline.

Saline.—An 0.85 per cent solution of sodium chloride containing 0.01 per cent phosphate buffer at pH 7.6 was used throughout.

Microcentrifuge Tubes.—In order to prepare uniform cell suspensions from small samples of blood, it is necessary to employ most of the cells in the sample, to be able to read the volume of packed cells accurately, and to transfer all of the measured cells to the suspending medium. As no centrifuge tubes were available that would readily fulfill these requirements, a procedure was devised that has proved highly satisfactory. Kahn 0.25 ml. pipettes were cut just beyond the bottom mark and then ground so that the end was even with this mark. They were cut near the upper end to make tubes 7 in. long. This end was slightly beveled on a grindstone and the lower end was beveled at an acute angle to allow it to reach the bottom of standard 15 ml. conical centrifuge tubes. Both ends were lightly fire polished. Heavy rubber bands to seal the ends of the tubes were prepared by cutting 1 in. strips of rubber from an automobile inner tube and securing the overlapping ends by means of numerous staples such as are commonly used for fastening papers.

Cell Suspensions.—After the last washing of the red cells, a microcentrifuge tube was passed to the bottom of the conical centrifuge tube containing the sedimented red cells and from 0.05 to 0.1 ml. of cells was allowed to flow in by hydrostatic pressure. The ends were sealed with the rubber bands and the tube was centrifuged at 1,000 r.p.m. for ten minutes. The volume of packed cells was read and the contents of the tube were rinsed into the appropriate volume of saline to make a 0.25 per cent suspension.

Tests.—All tests were performed in duplicate. Serial 1.5-fold dilutions of the stock virus suspension were prepared each day starting with 1:16. Equal volumes, 0.5 ml., of virus dilutions and cell suspensions were mixed vigorously. Employing the procedure recommended by the Naval Laboratory Research Unit No. 1,[19] the tubes were placed in the refrigerator for three to four hours, again shaken and allowed to stand for about seventeen hours at 4° C. The patterns of the sedimented cells were then observed and the highest dilution of virus that produced an even, salmon pink film was taken as the end point.

RESULTS

The concentration of virus in the high dilutions employed varied slightly from day to day because of changes in the stock suspension on storage and because of the pipetting errors inherent in preparing high dilutions so closely spaced as 1.5-fold steps. In the early tests performed in this series, the concentration of virus required to agglutinate human red cells was compared with that required to agglutinate the red cells of four adult chickens maintained as standards of reference. It soon became evident, however, that the vast majority of the human samples gave almost uniform results. The mean of the dilutions of virus required to agglutinate the six or more specimens that were tested in duplicate at one time was taken as the concentration required to agglutinate normal cells. This procedure is justified by the normal distribution found in the concentrations of virus required to agglutinate all but two of the samples of human

red cells. The results obtained with these two samples were not employed for determining the normal mean for the samples tested simultaneously with them.

The results of the 350 tests performed on forty-three presumably normal individuals and 295 patients giving similar results are shown in Fig. 1 together with the normal distribution curve for this number of samples. The chi square test for goodness of fit of this distribution to the normal curve gives a value

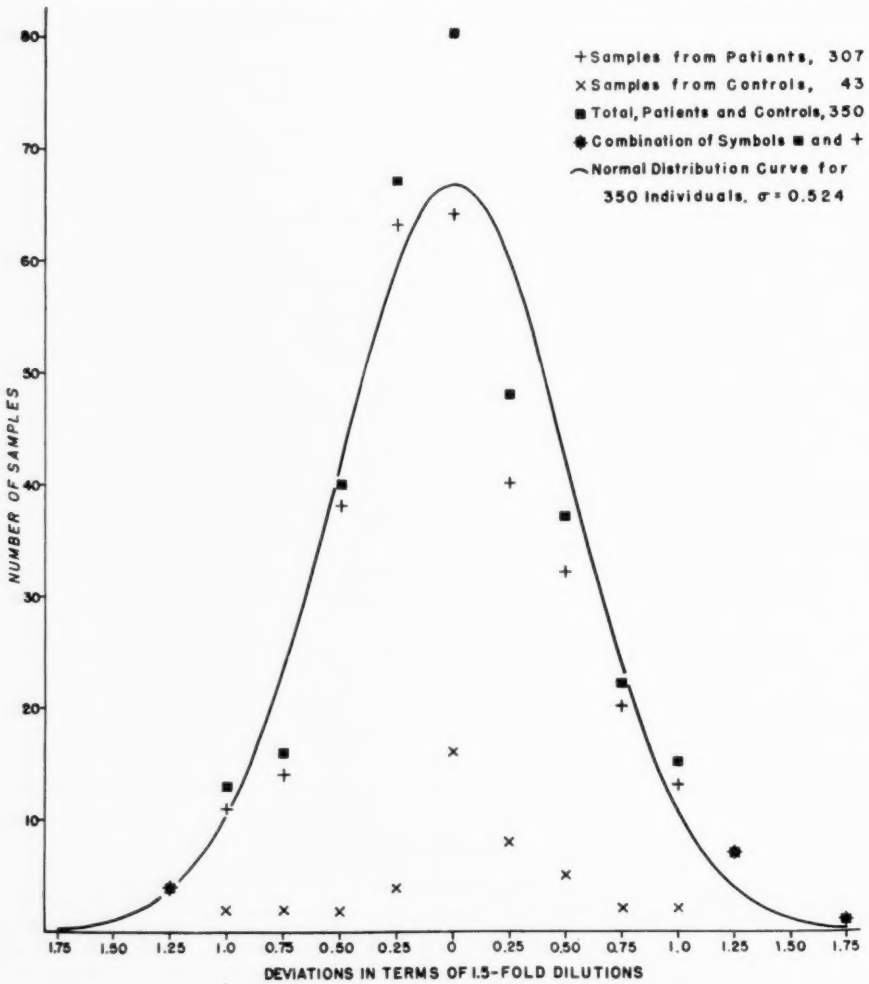

Fig. 1.—Distribution of the results of tests on the main group of samples.

for chi square of 13.67 with 7 degrees of freedom. A chi square as large as this may be expected to arise by chance in a normal distribution in 5 to 10 per cent of the times, so the distribution obtained may be accepted as normal. The statistical analysis presented in Table I indicates that no significant difference exists between the means of the results of the tests on erythrocytes from the controls and from all but two of the patients.*

*The author wishes to express his appreciation for assistance in the statistical analysis kindly furnished by Mr. M. H. Gordon, Clinical Psychologist at this hospital.

TABLE I. RELIABILITY OF DIFFERENCE BETWEEN MEAN END POINTS FOR PATIENTS AND CONTROLS

	PATIENTS		CONTROLS
Frequencies	307		43
Means	0.0106		0.0581
Standard deviations	0.5332		0.4472
Standard error of difference between means		0.0755	
t		0.6291	
p		0.47	

The major diagnoses of the 295 patients who furnished samples of red cells that gave results similar to the controls are summarized in Table II by a classification based on that contained in "Standard Nomenclature of Disease."[20] Among the cases diagnosed as being virus infections, there were eight cases of viral hepatitis and five cases of lymphocytic choriomeningitis.

Of the 352 samples of red cells tested, only two gave aberrant results. Both patients exhibited focal consolidations of the lungs. In one instance the lungs cleared in two weeks and the etiology could not be determined. Indications point to *Nocardia asteroides* as the etiologic agent in the other case. The concentrations of virus required to agglutinate these cells were, respectively, 3.4 and 7.6 times that required to agglutinate the cells in the homogeneous group derived from the other patients and from the normal controls. These concentrations represent 5.7 and 9.6 standard deviations from the mean of the homogeneous group. The chances in both instances, therefore, are less than one in a million that these results were due to the factors that caused a spread in the results of the tests on the samples of the main group. This variation in results was evanescent, since in each instance it was not found two days later and on subsequent test.

TABLE II. ETIOLOGIC CLASSIFICATION OF MAJOR DIAGNOSES

CAUSE OF DISEASE	NUMBER
Viruses and lower plant or animal parasites	150
Higher plant or animal parasites	11
Intoxication, including asthma	24
Trauma or physical agent	91
Circulatory disturbance	16
Disturbance of innervation or psychic control	42
Static mechanical abnormality	16
Disorder of metabolism, growth or nutrition	11
New growth	13
Unknown, structural reaction alone manifest	33
Unknown, functional reaction alone manifest	8
No disease manifest, controls	43

It has not been found possible to explain on the ground of technical error the transient resistance of cells to agglutination in these two cases. The virus dilutions gave the usual results with the five and seven samples tested at the same time. The duplicate tests gave identical results. Faulty cleaning of the tubes is most unlikely as several tubes were involved in each instance and the chances are very slight that they would have been selected in such a manner as to give the duplicate sharp end points noted. Also, in no instance in the series of tests

were irregular results noted that could be ascribed to improperly cleaned glassware. Apparent resistance would be observed if the cell suspensions were too dense, but this possible source of error was not present.

DISCUSSION

These results indicate a high degree of uniformity of human erythrocytes, whether from healthy individuals or from patients, in regard to their susceptibility to agglutination by the influenza A virus. In only two out of 309 samples tested from patients with a wide variety of conditions was a significant variation observed. The remaining 307 samples showed the same degree of agglutinability with influenza virus as did cells from the forty-three normal controls. In this homogeneous group of 350 samples that were tested in duplicate with serial 1.5-fold dilutions of virus, only 3.4 per cent of the averages of duplicate tests varied more than 1 tube from the mean end point of the group and none by as much as 2 tubes. The uniformity of results obtained with the samples from the normal individuals in this group confirms the observations of others.[19] Since human red cells are often used in hemagglutination inhibition tests for the determination of influenza antibodies, the uniformity of such cells in this reaction is of importance. The present study indicates that the chances are very slight that samples of red cells, even if obtained during illness, would vary significantly from one another in this regard.

When red blood cells are exposed in vitro to any of numerous substances, including certain bacterial toxins, the cells have been found to become refractory to agglutination by influenza virus. The results of the present series of tests on patients have failed to detect a similar action in vivo, with the possible exception of two cases in which a transient relative refractoriness to agglutination was observed.

SUMMARY

Red blood cells from 297 patients with a wide variety of conditions and from forty-three normal individuals were tested for their relative susceptibility to agglutination by influenza A virus. In all but two of the 352 tests performed, the red cells showed uniform susceptibility.

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THE OCCURRENCE OF ERYTHROBLASTOSIS FETALIS IN AMERICAN NEGROES

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THE possible correlation between the incidence of erythroblastosis fetalis and the distribution of Rh-negative blood in various races was originally suspected by Levine.[1] That erythroblastosis is very rare among Chinese, where the Rh-negative state is unusual, was shown by Levine and Wong.[2] Similar evidence for American Negroes has been only scanty. Potter,[3] in a large autopsy series of fetal and neonatal deaths, found erythroblastosis in 2.1 per cent and 0.7 per cent respectively of the white and colored infants. Out of 267 Negro infants studied, only two deaths were considered as due to erythroblastosis fetalis.

The disease has apparently been considered unusual in Negroes. Wiener and Wexler[4] published a series of three cases showing unusual symptoms of blood destruction in Negro infants but could not demonstrate any evidence of Rh sensitization. They remarked on the complete lack of reports of such cases in Negroes and on the basis of their observations suggested that some obscure blood factor might be responsible.

It is probably true that the obstetric course of many pregnant Negroes is not followed by a physician, and presuming that cases of erythroblastosis did occur, they would go undiagnosed and hence unknown. Furthermore, from a clinical viewpoint minimal degrees of jaundice are not obvious in Negro infants and might go undetected. Recently Waller and Waller[5] presented data on several cases of Rh immunization in Negroes with substantial laboratory evidence to support their findings. Our experience is similar to theirs and covers a period of several years. Up to the present time we have seen twelve cases of immunized Rh-negative Negroes, and because of the scarcity of reports of this disease in this race, I am presenting these cases in some detail.

METHODS

All D (Rh_o) blood typings were done by the warmed slide technique using at least two different anti-D typing serums. Our technique for Rh antibody titrations is patterned after that first suggested by Abelson and elaborated upon and published by Diamond and Denton.[6] This is the widely employed technique using doubling dilutions of the patient's unknown serum with saline, addition of small quantity of a 2 per cent saline suspension of Rh-positive red cells to an equal quantity of each dilution, incubation, and reading. The saline supernate is then replaced with a few drops of a 1:1 mixture of AB serum (Hyland) and 30 per cent bovine albumin (Armour) and again read. Each unknown serum was first screened for Rh antibodies by setting up with two different Rh-positive cells (phenotypes CDe and cDE) before actually titrating the serum. An Rh-negative control cell (cde/cde) was carried

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through the method each time and consistently showed no agglutination in either saline or serum-albumin medium. Details of the titration technique employed have been published elsewhere.[7]

The diagnosis of erythroblastosis fetalis was made on both clinical and serologic grounds. A cord blood sample was always taken at every Rh-negative delivery, and where the mother had been showing antibodies various laboratory tests were done routinely. While the classical symptoms of erythroblastosis were looked for in all cases, clinical symptoms in this disease are notoriously misleading, particularly immediately after delivery. In some of the milder cases the laboratory findings alone were relied upon to make the diagnosis. In all instances a diagnosis was made only when the mother showed antibodies to a red cell antigen which was present in her husband but lacking in her blood, and where the infant was shown to possess this antigen. Furthermore, the diagnosis was not made until a positive Coombs test had confirmed the foregoing data. The Coombs test has proved to be an excellent diagnostic aid. It confirms in a single maneuver not only the maternal-fetal blood group incompatibility and maternal immunization, but proves that the infant's red cells are coated with antagonistic antibodies. The number of nucleated red cells on the smear and the icteric index have been of questionable diagnostic value, particularly in the milder cases of the disease.

CASE REPORTS

CASE 1.—Mrs. L. H. was a 23-year-old Negress who delivered a normal child in January, 1944. Following this delivery, the patient received a blood transfusion from a donor who recently was shown to be type O, Rh positive.

The child of her second pregnancy was a girl, born in November, 1945. The infant was apparently normal except for slight jaundice. She weighed 3,680 grams and was type O, Rh positive. Feeding was on breast and formula. On the third day the infant became severely jaundiced and the icteric index was 98. There was slight edema and the liver and spleen were both enlarged and easily palpated. The hemoglobin was 14 Gm., red cell count 3.8 millions, and there were 18 erythroblasts per 100 white cells. A diagnosis of erythroblastosis fetalis was made. Treatment consisted of two transfusions with 50 ml. compatible Rh-positive blood which brought the hemoglobin to 14.7 Gm. and the red cell count to 4.4 millions. By the fourth day no nucleated red cells were seen on the smear, although on the sixteenth hospital day the hemoglobin had dropped to 9.6 Gm. and the red cell count to 3.2 millions.

Her third child was delivered in October, 1948. A cord blood specimen was type O, Rh positive. The infant weighed 4,000 grams. The icteric index was 5, hemoglobin 13.5 Gm., and red cell count 4.7 millions. There were 29 nucleated red cells per 100 white cells. There was no edema; there was mild icterus; the tip of the spleen was just palpable, and the liver was down one finger. A blood specimen was taken from the mother for Rh antibody titration which showed an end point at 1:32, and a diagnosis of erythroblastosis was made. A replacement transfusion was performed. The hemoglobin remained near 14 Gm. and by the tenth day the smear showed only 3 nucleated red cells per 100 white cells. The infant was discharged in good condition. A summary of the blood grouping data on this case is given in Table I.

TABLE I

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			0	–	–	–	+	cde/cde
Father			0	–	+	+	+	cDE/cDe
Sister (blood donor)			0	–	+	–	+	cDe/cDe or cDe/cde
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	0	–	+	+	+	cDE/cde
2	Term	Severe EB	0	–	+	+	+	cDE/cde
3	Term	Moderate EB	0	–	+	–	+	cDe/cde

EB, erythroblastosis.

CASE 2.—Mrs. T. I. was a Negress whose first four children were delivered at home and no information was available regarding their neonatal condition. Her fifth child was born in June, 1947, and showed jaundice on the second day which persisted about two weeks. The urine during this period was dark yellow. The child was type A, Rh positive. The mother was found to be type A, Rh negative for the C, D, and E factors. An Rh antibody titration showed a one-plus agglutination end point at 1:128 in saline and 1:64 in 20 per cent bovine albumin. The microscopic end points were one dilution higher in each medium. Almost identical end points were found on a repeat titration two months later.

Her sixth child was delivered at another hospital where she was mistakenly thought to be Rh positive. The infant was an apparently normal female child and was put on breast feeding. At 3 days of age the infant became jaundiced; the abdomen was moderately distended and the nurse reported that the urine was deep yellow. The condition of the infant was unusual but no diagnosis could be made. The infant and mother were discharged on the sixth day. The infant was breast fed throughout and when 4 weeks old was readmitted to the hospital because of increasing irritability, marked jaundice of the skin, scleras and mucous membranes, and failure to nurse well.

On admission, respirations were irregular and rapid. The icteric index was 176. The stools were brown; the urine showed positive tests for bile and had an elevated urobilinogen. The liver was palpated two fingers down, while the spleen was not palpable. The infant was acutely ill and showed symptoms of air hunger. Therapy consisted of whole blood transfusions, iron supplements to formula, and so on. The infant began to show some improvement and after eighteen days the hemoglobin was 12 Gm. and the red cell count was 4.7 millions. The icteric index was 62. There was still some jaundice, but the infant was much improved. The mother's Rh type was rechecked and she was found to be Rh negative. The infant was type B, Rh positive. No anti-Rh tests were done, but on the strength of the Rh incompatibility and the clinical findings, a belated diagnosis of erythroblastosis fetalis was made. The infant was discharged on iron therapy when 8 weeks of age.

The patient's seventh pregnancy was followed in our clinic with Rh antibody titrations. The end points in serum-albumin were 1:64, while the titer in saline rose from 1:1 to 1:32 just before term. The infant was delivered on Sept. 10, 1949, by cesarean section at 39 weeks because of the rising titer. The placenta was somewhat enlarged but pathologic examination later showed no changes of the erythroblastotic type. The infant was a boy weighing 3,460 grams and was type AB, Rh positive, with a positive Coombs test. The liver was down one fingerbreadth and the spleen was just palpable. The icteric index was 45, hemoglobin 14 Gm., white cell count 14.9 thousands, and red cell count 4.3 millions. A smear showed 19 nucleated red cells per 100 white cells. The infant was given a replacement transfusion with 510 c.c. of type O, Rh-negative blood. On the following day, blood counts showed a hemoglobin of 14 Gm., red cell count 3.4 millions, and a white cell count of 17,800.

TABLE II

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			A	–	–	–	+	cde/cde
Father			O	–	+	–	+	cDe/cDe (probably)
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	O	–	+	–	+	cDe/cde
2	Term	Normal	O	–	+	–	+	cDe/cde
3	Term	Normal	A	–	+	–	+	cDe/cde
4	Term	Normal	AB	–	+	–	+	cDe/cde
5	Term	Mild EB	A	–	+	–	+	cDe/cde
6	Term	Severe EB	B	–	+	–	+	cDe/cde
7	Term	Mild EB	AB	–	+	–	+	cDe/cde

The presence of the B antigen in the fourth, sixth, and seventh child proves illegitimacy.
EB, erythroblastosis.

The hemoglobin remained between 13 and 14 Gm. and the infant was discharged on the tenth day. When seen in the Clinic at 20 days of age the infant was progressing normally but was somewhat pale. The spleen and liver were still palpable. The hemoglobin was 9.5 Gm. at this time. The blood grouping data on this family are summarized in Table II.

CASE 3.—Mrs. L. F. was a 22-year-old Negress who delivered a normal infant in November, 1941. Her second pregnancy resulted in delivery of an apparently normal boy in January, 1943. The next pregnancy ended in a miscarriage in the third month. For her fourth pregnancy she was delivered of twin boys in July, 1944. The heartbeat in one fetus disappeared just before delivery and this infant was stillborn while the second was apparently normal. The fifth child was delivered in July, 1945. The infant was full term, without malformations, and weighed 2,709 grams. The placenta was moderately edematous. There was mild jaundice at birth but the infant became severely jaundiced on the second day. Respirations became labored with periods of apnea. The infant became limp and cyanotic. The hemoglobin was 7.0 grams. The liver and spleen were not palpable and the scleras were very icteric. The infant was given emergency measures but finally expired on the fifth day. No Rh studies had been done, but an autopsy showed generalized icterus with bile pigment stains throughout the brain. There was moderate erythropoiesis of the liver and spleen. The final pathologic diagnosis was typical erythroblastosis fetalis with kernicterus.

This patient became pregnant for the sixth time and because of her history Rh studies were performed. She was Rh negative and her serum contained saline and serum-albumin active Rh antibodies. She was delivered in February, 1947, of a term female infant. The placenta was apparently normal. The infant weighed 3190 grams. There was no icterus, no edema and no enlargement of the liver or spleen. The hemoglobin was 17.5 Gm. and the red cell count 6.5 millions on the eleventh day of life. The infant was breast fed and sent home in good condition. Recent blood studies on this child showed her to be type O, Rh positive of the probable genotype combination CDe/cde. It is interesting that this Rh-positive child showed no symptoms of erythroblastosis although at least one previous child had died from the disease. Similar cases have been reported in the literature in white patients however. Furthermore, this patient had three living children born previous to the pregnancy.

The patient was delivered of her seventh child in April, 1949, by section at 37 weeks because of her history, together with the elevated Rh antibody titer. This had shown a rise from 1:8 to 1:64 in saline just before term. The serum-albumin titer remained at 1:32 until post partum when it rose slightly. The infant was a girl and weighed 2,790 grams. A cord blood specimen was type A, Rh positive. The Coombs test was strongly positive. The infant showed mild jaundice. The liver was just palpable one finger down and the spleen was not palpable. The hemoglobin was 13.5 Gm. and the red cell count 4.5 millions.

TABLE III

BLOOD FROM			ОАВ GROUP	RH SUBGROUPING				GENOTYPE
				C	D	E	c	
Mother			A	–	–	–	+	cde/cde
Father			A	+	+	+	+	CDe/cDE or CDe/cDe ?
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	A	+	+	–	+	CDe/cde
2	Term	Normal	O	+	+	–	+	CDe/cde
3	Miscarriage		No data					
4	Term twin	Normal	A	–	+	–	+	cDe/cde
	Term twin	Stillborn	No data					
5	Term	Severe, died 5th day	No data					
6	Term	Normal?	O	+	+	–	+	CDe/cde
7	37 wk.	Moderate	A	+	+	–	+	CDe/cde

There were 4 nucleated red cells per 100 white blood cells. A diagnosis of erythroblastosis fetalis was made and a replacement transfusion performed. The hemoglobin remained up and the infant was discharged when 14 days of age. Repeated typings on the husband confirmed the presence of the E (Rh″) factor with a probable genotype combination CDe/cDE. None of the children inherited the E substance and yet both the cDe and CDe combinations occurred which suggests illegitimacy. The blood grouping data on this case are detailed in Table III.

CASE 4.—Mrs. O. M. B. was a Negress with a ten-year history of chronic gastrointestinal bleeding in addition to her Rh immunization. Her first child was a normal female infant born in March, 1939. Because of the patient's persistent vomitting of coffee ground material during this pregnancy and her severe hemorrhage at delivery, she received a blood transfusion which was well tolerated. She was transfused again three days later and had a fairly severe reaction, with a temperature elevation to 40.4° C. accompanied by a severe chill. Her second infant was delivered at home in April, 1941. The infant was approximately of 36 weeks' gestation and was severely jaundiced. This child lived only three days. On the day of the infant's death, the mother noticed bloody material in the mouth and "bruised spots under the skin." No autopsy was performed but death was undoubtedly due to erythroblastosis complicated by the hemorrhagic diathesis.

The third pregnancy resulted in a miscarriage at three months in June, 1942. Her fourth pregnancy resulted in a stillborn 8-month male fetus delivered in February, 1943. Fetal movements had ceased about three days before the patient went into spontaneous labor. The infant was stillborn and weighed 3 pounds, 13 ounces. The placenta weighed 1 pound, 12 ounces and the baby to placenta weight ratio was 2.2:1. There was generalized severe edema and an autopsy revealed multiple petechial hemorrhages. Blood from the baby was taken at this time and a cross-match set up with the mother's blood was incompatible. A blood smear showed numerous nucleated red blood cells. There was marked myeloid metaplasia of the liver. The placenta was large and edematous and the final pathologic diagnosis was erythroblastosis fetalis.

During this last pregnancy the patient vomitted blood and was in impending shock when admitted in labor. A transfusion was performed using her sister as donor, who has recently been shown to be Rh positive. Her condition remained critical and blood chemistry studies showed blood urea nitrogen of 80 and an icteric index of 87. At the time the nitrogen retention and jaundice were not satisfactorily explained, but they were undoubtedly symptoms of a transfusion reaction. No Rh determinations were done but it was found that the patient's serum weakly clumped the infant's cells. It was also shown that the mother's blood partially clumped the donor's cells. An Rh antibody titration done recently on this patient (six years post partum) showed a trace agglutination at 1:2 in saline medium and a trace at 1:128 in serum albumin. The father was not available for study. The blood grouping data on this case are summarized in Table IV.

TABLE IV

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			O	−	−	−	+	cde/cde
Sister (blood donor)			O	−	+	−	+	cDe/cDe or cDe/cde
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	A	−	+	−	+	cDe/cde
2	36 wk.	Severe EB, lived 3 days	No data					
3	13 wk.	Miscarriage	No data					
4	36 wk.	Severe EB, stillborn	No data					

EB, erythroblastosis.

CASE 5.—Mrs. O. M. R., a 19-year-old Negress, was delivered of her first child Aug. 8, 1948. This child is alive and well and recently was shown to be Rh negative. During her second pregnancy she was found to be type A, Rh negative. Rh antibody titers were positive at 1:4 in saline and 1:8 in serum-albumin just prior to term. She was delivered Sept. 13, 1949, of an apparently normal male infant of the calculated 38 weeks of gestation. The child weighed 3,260 grams and was type A, Rh positive. The cord blood Coombs test was positive. The infant showed no icterus, no edema, and no enlargement of the liver or spleen. The hemoglobin was 13 Gm., red cell count 3.2 millions, and white cell count 15,000.

Because of the positive Coombs test and the known maternal immunization, the infant received a replacement transfusion. The hemoglobin rose to 17 Gm. and the red cell count to 5.9 millions. On the day following the replacement the hemoglobin was 15 Gm. and the red cell count 4.6 millions. When the child returned to the Clinic three weeks later the hemoglobin was only 9 Gm. and the red cell count 3.2 millions. Physical findings were otherwise negative and the infant was alert and gaining weight. The blood grouping data on this family are presented in Table V.

TABLE V

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			A	–	–	–	+	cde/cde
Father			A	+	+	–	+	CDe/cde
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	O	–	–	–	+	cde/cde
2	38 wk.	Normal?	A	+	+	–	+	CDe/cde

CASE 6.—Mrs. G. G. was a 36-year-old Negress pregnant for the third time with no history of transfusion. Her first two children were apparently normal at birth. This third pregnancy was sixteen years after the second and there were no routine Rh studies undertaken. The baby was delivered in October, 1948, and was a moderately jaundiced male infant. The birth weight was 2,200 grams. There was inadequate respiration and profound cyanosis, and death occurred in twenty minutes. A cord blood specimen was type O, Rh positive. A blood specimen was taken from the mother and examined for Rh antibodies. Known Rh-positive cells were clumped in saline to a one-plus end point at 1:256, and in 20 per cent bovine albumin to a one-plus end point at 1:1024. Microscopic end points were one dilution tube higher in each case. The infant was autopsied and the lungs were nonbuoyant. The blood was confirmed as Rh positive. There was no edema of the extremities, but there was generalized icterus including the abdominal organs. Microscopically there was marked extramedullary hematopoiesis of the liver, spleen, thymus, stomach, adrenals, and bile pigment in the liver cells. The liver was not enlarged but the spleen weighed 23 grams. The brain showed no evidences of edema, hemorrhage, or congenital deformity. There was no evidence of kernicterus. Besides the profound icterus and atelectasis, there

TABLE VI

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			B	–	–	–	+	cde/cde
Father			A	–	+	–	+	cDe/cde
PREGNANCY	RESULT	CONDITION						
1	Term	Normal	A	–	+	–	+	cDe/cde
2	Term	Normal	No data					
3	Term	Stillborn, severe EB	O	–	+	–	+	cDe/cde
4	Term	Normal	B	–	–	–	+	cde/cde

EB, Erythroblastosis.

were diffuse hemorrhages into the lungs, adrenals, thymus, spleen, and intestines. There were petechiae of the skin, epicardium, and lungs. The final pathologic diagnosis was erythroblastosis fetalis, with bilateral fetal atelectasis as the immediate cause of death.

Two weeks post partum the anti-Rh titration was repeated and showed microscopic end points at 1:32 in saline and at 1:256 in 20 per cent bovine albumin. Later about 400 ml. of blood were obtained from this patient. This serum, following suitable preparation, served as a satisfactory reagent for routine Rh typing. It was apparently pure D (Rh_o) antibody and in conjunction with several commercially available anti-Rh_o (D) typing serums showed only a rare discrepancy in over 3,500 duplicate blood typings. The specificity was later confirmed by finding the husband negative for the C and E factors and only positive to D. This patient's fourth pregnancy was followed closely with Rh antibody titrations. The titer in saline remained at 1:4 while the serum-albumin antibody titer fell from 1:128 to 1:32 as the patient approached term. The infant was delivered on Jan. 10, 1950, and was clinically normal. A cord blood was type B, Rh negative with a negative Coombs test. There were only 6 nucleated red cells per 100 white cells, with an icteric index of 12. The hemoglobin was 21.5 Gm., red cell count 6.8 millions, and white cell count 17,000. The blood grouping data on this case are summarized in Table VI.

CASE 7.—Mrs. D. W. was an 18-year-old Negress who delivered a normal child in October, 1943. Her next three pregnancies all resulted in intrauterine fetal death before term. The fifth pregnancy resulted in fetal death at about 17 weeks. An autopsy of the fetus contributed little information because of the extensive maceration. At this time the patient's blood was typed and found to be type O, Rh negative. During the sixth pregnancy Rh antibody titrations were done for the first time. Several titers showed end points at 1:128 in serum-albumin. There was almost no activity in saline medium. X-rays showed overriding of the cranial bones and on Nov. 25, 1949, the patient delivered an extensively macerated fetus. The infant was approximately of 24 weeks' gestation and had been dead about seven weeks. An autopsy was unsatisfactory because of the autolysis. The blood grouping data on this case are detailed in Table VII.

TABLE VII

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			O	−	−	−	+	cde/cde
Father			O	+	+	−	+	CDe/cDe or CDe/cde
PREGNANCY	DURATION	CONDITION						
1	Term	Normal	O	+	+	−	+	CDe/cde
2	Term	Stillborn	No data					
3	36 wk.	Stillborn	No data					
4	36 wk.	Stillborn	No data					
5	17 wk.	Miscarriage	No data					
6	31 wk.	Stillborn, severe EB	No data					

EB, Erythroblastosis.

CASE 8.—Mrs. M. A. was a 15-year-old Negress delivered of her first child April 19, 1946. This child is alive and well. She was delivered of her second child on Aug. 17, 1949. During this pregnancy she was found to be Rh negative and anti-Rh titrations were positive. At about 34 weeks her titer was 1:2, rising to 1:8 both in saline and serum-albumin just before delivery. The infant was an apparently normal term female. There was no edema, no icterus, and the liver was just palpable. The placenta was normal in appearance. Laboratory examination of the cord blood showed the infant to be type O, Rh positive, with a positive Coombs test. The icteric index was 13, hemoglobin 17 Gm., red cell count 5.5 millions, and white cell count 5,500. There were 7 nucleated red cells per 100 white cells. Because

TABLE VIII

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			B	–	–	+	+	cdE/cde (probably)
Father			O	–	+	+	+	cDE/cDe
PREGNANCY	RESULT	CONDITION						
1	Term	Normal	O	–	+	+	+	cDE/cdE or cDE/cde
2	Term	Normal	O	–	+	–	+	cDe/cde

of the positive Coombs test, a replacement transfusion was performed. The child remained asymptomatic and was discharged. The blood grouping data in this case are summarized in Table VIII.

CASE 9.—Mrs. S. H. was a 24-year-old Negress delivered of her first child Oct. 30, 1944. The infant was an apparently normal term female. Recent blood studies have shown this child to be Rh negative. This patient subsequently delivered two normal infants at home. During her fourth pregnancy she was found to be Rh negative and three Rh antibody titrations done prior to delivery showed one-plus end points at 1:16 in serum albumin. The saline agglutinins never showed more than trace activity in full strength serum. She was delivered July 21, 1949. The infant was an apparently normal male. There was no jaundice and there were no clinical symptoms of erythroblastosis. Blood counts showed 18.5 Gm. hemoglobin, red cell count 6.4 millions, and white cell count 8,500. The smear was not unusual. The infant was discharged in good condition with the mother on the third day. The blood grouping data on this case are detailed in Table IX.

TABLE IX

BLOOD FROM			ABO GROUP	RH SUBGROUPING C	D	E	c	GENOTYPE
Mother			O	–	–	–	+	cde/cde
Father			O	–	+	–	+	cDe/cde
PREGNANCY	RESULT	CONDITION						
1	Term	Normal	O	–	–	–	+	cde/cde
2	Term	Normal	O	–	+	–	+	cDe/cde
3	Term	Normal	O	–	+	–	+	cDe/cde
4	Term	Normal	O	–	+	–	+	cDe/cde

CASES 10, 11, and 12.—These three patients were Rh-negative Negroes who were definitely immunized to the Rh factor by careful laboratory titrations. Each was the typical Rh-negative genotype (cde/cde). The husbands and children either were not available for study or there was admitted illegitimacy and the data are incomplete.

DISCUSSION

In this paper the Fisher-Race terminology and genetic theory are employed preferentially. When the term "Rh positive" is employed without qualification it indicates the presence of the D (Rh_o) factor. Since man has paired chromosomes the corresponding gene may also be Rh positive, making the individual "homozygous." He is "heterozygous" if the D (Rh_o) factor is not present on both sides. If the D (Rh_o) factor is not present there is a corresponding gene called "little" d, Hr_o or the "Hr factor" present instead. Thus the individual may be DD, dd, or Dd. The same relationship obtains with the C (Rh′) and

the E (Rh″) factors. Thus at least thirty-six different gene combinations are theoretically possible, and as additional gene variants are discovered the number of possible genotypes increases proportionately. This system of genetic terminology lends itself well to Rh family studies.

Most of the cases presented here are well known to us and have been followed through one or more pregnancies. Eleven of the twelve Rh-immunized Negro women were the typical Rh-negative type, i.e. they lacked the C, D, and E factors. The one exception was an unusual combination of the cdE factors. Each blood was positive to the "little" e (Hr′) factor, and presumably also to the d (Hr_o) and e (Hr″) factors although the presence of these latter two cell substances was not checked because of lack of the specific typing serums. The Rh antibodies found in their serum were apparently the same as found in American whites. The agglutination reactions with Rh-positive cells from white donors were clear cut and not unusual. Serum from two of these Negro patients has been used as a laboratory anti-D typing reagent in conjunction with commercially available typing serums and the reactions have been in almost perfect agreement. Specificity tests on the serums used as laboratory typing reagents showed pure anti-D activity. This was later confirmed by finding the gene combination cDe in the husbands and children of these women.

Prior to November, 1948, we recognized six immunized Negro women who had each delivered at least one erythroblastotic infant. These patients were discovered after a clinical diagnosis of erythroblastosis had been made on one of their offspring and their sensitization was corroborated by careful laboratory tests. Since then we have detected six more immunized, Negro, Rh-negative women of whom five had no suggestive history.

In the past year we have carried in the Maternity Clinic about 2,200 Negro patients of whom over 1,400 have been delivered in the hospital. With a few exceptions these patients have been screened for the Rh factor, but unfortunately all the negative patients were not checked for Rh antibodies. All patients with suggestive histories were checked carefully however. Out of fifty-two Rh-negative patients who were titered solely because they were Rh negative (excluding known and probable cases of immunization), we detected Rh antibodies in six individuals giving an incidence of immunization of about 11.5 per cent. During the same period of time an unselected series of white Rh-negative patients was examined for Rh antibodies and among 493 such random blood samples 48 were found with Rh antibodies. This gives a percentage (10 per cent) which is similar to that found among the Negroes. Clinically only two or three of the Negro cases might have been diagnosed out of the year's deliveries, while on a serologic basis the incidence of erythroblastosis would be higher. Prior to November, 1948, we recognized six cases among 4,200 deliveries during a three-year period, giving an incidence of erythroblastosis in Negroes of about 1:700 deliveries. On our Private Service which is almost entirely white we have observed 27 cases of erythroblastosis among 8,902 deliveries over the same three-year period, giving an incidence of about 1:330 deliveries. While these bald figures are admittedly subject to error, they seem to indicate that the incidence of erythroblastosis in whites is approximately double that in Negroes.

The general severity of the erythroblastosis in the offspring of these immunized Negroes was not apparently different from the condition in whites. Out of the nine cases on which complete data were available, two patients had a total of two living children (the first born to each) of a total of ten pregnancies. Out of the remaining eight pregnancies of these two patients there were five stillbirths, one neonatal death, and two miscarriages, perhaps induced. Various titers on one of these patients showed end points near 1:256, while a single titer on the second patient done six years post partum (Case 4) was 1:128. Four other patients with a total of twenty term pregnancies had nine infants affected with some degree of erythroblastosis. Their highest titers were 1:64, 1:64, 1:128, and 1:256.

On the other hand the three remaining patients who were definitely immunized had a total of eight offspring who showed minimal or no clinical symptoms of erythroblastosis and who are all alive and well. Possibly clinical symptoms did not develop in the last born of two of these patients because a replacement transfusion was purposely carried out immediately to forestall such a possibility. It is interesting that no titer done during the last pregnancy of these three patients exceeded 1:16, and indeed most of the titers were 1:8 or 1:4 with significantly weak saline activity. The correlation between the antibody titer end point and the clinical severity of the erythroblastosis was quite satisfactory. The mothers of the mildly affected infants in general carried low titers with little evidence of fresh stimulation as indicated by the weak "early immune" saline type antibody titer.

Waller and Waller[5] reported that only one case out of their series of eight immunized Rh-negative Negroes ever showed any saline-active Rh antibodies. Five of their patients who were delivered had infants normal clinically. In our series of twelve cases, seven patients showed saline-active agglutinins ranging in titer from 1:4 up to 1:512. The remaining five cases were made up of Case 4 in which the titer was done six years post partum, Case 7 in which another stillbirth was pending, and Cases 5, 8, and 9 in which the infants were only mildly affected.

BLOOD GROUP FREQUENCY

Over the past year we have carried out 2,530 consecutive blood typings for the A, B, and D (Rh_o) factors on Maternity Out Patient Department Clinic patients. This total includes 2,200 Negroes and 330 whites. Although the specimens from white patients total only 330, these particular blood specimens were taken along with the Negro specimens and therefore should serve as a control series. The race of the patient was not known in the laboratory prior to the blood grouping determination. Among the whites the percentage of Rh positives was 87.0 per cent. This figure is in the generally accepted range for random white blood samples and is similar to the percentages found in this laboratory for larger series of unselected white specimens.

The percentage of Rh positives found in the series of 2,200 Negroes was 93.3 per cent and the data are tabulated in Table X. There was apparently an even distribution over each of the International blood groups and the over-all

percentage of Rh positives, 93.3 per cent, agrees closely with frequency figures found by Levine,[8] 92.6 per cent, Tisdall and Garland,[9] 90.1 per cent, and Waller and Waller,[5] 92.7 per cent.

Recently Santos and Stepto[10] reported an incidence of 85 per cent Rh positives in a series of 1,000 Rh_o (D) typings on Negroes. Their figure is not different from that usually accepted for the Caucasian population and is at variance with the data of other investigators.

TABLE X. FREQUENCY OF OAB AND Rh_o (D) BLOOD GROUPS IN 2,200 AMERICAN NEGROES

INTERNATIONAL GROUP	PERCENTAGE (BOTH RH + AND −)	RH POSITIVE	RH NEGATIVE	PER CENT RH POSITIVE
O	42.2	976	62	94.0
A	27.1	547	49	91.8
B	22.7	468	32	93.6
AB	3.0	61	5	92.4
Total patients		2,052	148	93.3

Our findings for the frequency of the OAB groups in Negroes confirm the data of previous investigators including Wiener, Belkin, and Sonn.[11] The American Negro apparently shows a higher incidence of groups O and B and a lower incidence of group A. The corresponding figures of the O, A, B, and AB groups on the short series of 330 whites were 39.4, 41.8, 15.2, and 3.0 per cent respectively. Larger series on whites done in this laboratory show approximately the 45, 41, 11, and 3 per cent distribution usually accepted for American whites.

While intermarriage with whites has been suggested as a possible explanation for the intermediate Rh-positive percentage found in Negroes, such an explanation is inadequate in explaining the high incidence of the "pure" D (Rh_o) state in this race. This is the genotype combination in which the individual is "Rh positive" to the Rh_o or D factor but negative for the Rh′ (C) and Rh″ (E) factors (cDe). Such a genotype combination occurs only about 2 per cent of the time in random whites but is common among Negroes. Levine[8] reported an incidence of 46 per cent for this "pure" Rh_o (D) combination among Negroes. Among the husbands of the immunized Negro patients presented here, it was demonstrated that nine men had the "pure D" genotype combination cDe on at least one of their chromosomes. Three men were proved to be heterozygous positives by the finding of Rh-negative offspring. Because of this independent occurrence of the C and D factors, the routine determination of zygosity of Negro husbands is impossible at present. Actually no zygosity test is accurate unless the blood is tested against antiserum specific for the d (Hr_o) factor, but since this particular antiserum is usually not available, zygosity is determined for C (Rh′) factor and then the zygosity of the clinically important D factor is assumed. Such a procedure is valid in whites because the C and D factors occur together quite commonly. Mollison, Mourant and Race[12] have discussed the errors inherent in such a procedure. When this technique is applied to Negro blood, however, the errors involved become significant.

SUMMARY

1. Twelve cases of immunization to the Rh factor in American Negroes have been presented.

2. The Rh blood group incompatibilities, Rh antibodies, and the clinical symptoms of erythroblastosis fetalis in the offspring were all apparently identical to those occurring in American whites.

3. The frequency of occurrence of the red cell antigens A and B, and the Rh factor D (Rh_o), in American Negroes living in Cleveland, Ohio, is reported.

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THE LIVER IN PNEUMOCOCCAL PNEUMONIA: OBSERVATIONS IN 94 CASES ON LIVER FUNCTION AND JAUNDICE IN PNEUMONIA

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WHILE the occurrence of jaundice in pneumonia has been recognized for more than a century,[1, 2] its pathogenesis has remained uncertain. Numerous studies of this condition appeared in the older literature,[3-6] but there have been few in recent years. Although there have been adherents to the opinion that the jaundice is hepatocellular, or even obstructive, most workers have considered it hemolytic in origin. Certain studies, however, using some of the earlier tests of liver function which showed consistent hepatocellular damage in pneumonia, as well as the reports of bile in the urine and direct-reacting bilirubin in the blood, are clearly suggestive of a hepatocellular origin of the jaundice. Because such evidence appears to be of critical importance in evaluating the concept of a primary hemolytic origin of the jaundice, it was decided to reinvestigate the status of liver function in pneumonia with the use of modern techniques, and to study the pigment disturbance in patients with pneumonia and jaundice. We have attempted a critical review of the literature in order to coordinate previous work into a rational concept of the pathogenesis of jaundice in pneumonia, and we have added certain observations of our own.

METHODS AND PROCEDURES

Eighty-one patients with pneumococcal pneumonia without clinical jaundice were selected at random during the winter of 1947-1948 for study. In seventy-one of these patients, liver function studies were done within forty-eight hours after admission to the hospital. In the remaining ten these studies were performed between the second and fifth hospital day. In thirty of the patients whose functions were studied during the first two days, these tests were repeated during convalescence. In addition, thirteen patients with clinical jaundice were studied from the standpoint of determining the nature of the jaundice. Bacteriologic studies of the sputum were done in sixty-five of the patients without jaundice and in nine of the patients with jaundice. Records of blood culture were available for forty-seven of the nonjaundiced patients and for six of those with jaundice.

The tests of liver function employed in all the patients were bromsulfalein excretion, thymol turbidity, and cephalin-cholesterol flocculation. The bromsulfalein test was performed according to the modification of Mateer's group,[7] (using the 5 mg. per kilogram dose) the degree of dye retention being determined spectrophotometrically at the end of 45 minutes. Retention of 4 per cent or more at the end of 45 minutes was regarded as abnormal. The thymol turbidity test was done by the method described by Maclagan,[8] using the modification of Shank and Hoagland[9] for the spectrophotometer. In this laboratory 4 units represents the upper limit of normal. The cephalin-cholesterol flocculation test was carried out according to Neefe's[10] modification of the procedure of Hanger.[11] A reading of 3 plus or 4 plus in 48 hours was considered positive.

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Serum bilirubin in the patients with jaundice was determined according to the procedure of Malloy and Evelyn.[12] Urine bile was demonstrated by Gmelin's test[13] or by the modified Harrison spot test.[14] Urine was tested for urobilinogen either by the method of Wallace and Diamond,[15] with the upper limit of normal being a positive reaction in a 1 to 20 dilution, or by Watson's[16] simplified procedure, with the upper limit of normal being 1.4 Ehrlich units.

RESULTS

Table I(A) summarizes the data on eighty-one patients without clinical jaundice. It can be seen that sixty-six (81 per cent) showed a BSP retention of more than 4 per cent on admission, with a mean retention of 12.8 per cent.

TABLE I. LIVER FUNCTIONS IN ACUTE BACTERIAL PNEUMONIA

(A) RESULTS IN ENTIRE SERIES OF 81 PATIENTS

LIVER FUNCTIONS*	NUMBER ABNORMAL	PER CENT	MEAN
BSP	66	81.5	12.8 ∓ 1.9
T. T.	17	20.9	3.2 ∓ 0.4
C. F.	59	72.8	--------

(B) RESULTS IN 71 PATIENTS TESTED DURING THE FIRST 48 HOURS IN THE HOSPITAL

LIVER FUNCTIONS	NUMBER ABNORMAL	PER CENT	MEAN
BSP	62	87.3	14.3 ∓ 1.7
T. T.	15	21.1	3.3 ∓ 0.5
C. F.	53	74.6	--------

(C) RESULTS IN 30 PATIENTS DURING THE ACUTE AND CONVALESCENT STAGES

	ON ENTRY			DURING CONVALESCENCE		
	ABNORMAL			ABNORMAL		
LIVER FUNCTION	NUMBER	PER CENT	MEAN	NUMBER	PER CENT	MEAN
BSP	28	93.3	18.4 ∓ 1.7	8	26.6	4.7 ∓ 1.1
T. T.	9	30.0	3.9 ∓ 0.5	7	23.3	3.7 ∓ 0.4
C. F.	24	71.0	--------	16	53.3	-------

*BSP, Bromsulfalein excretion.
T. T., Thymol turbidity test.
C. F., Cephalin-cholesterol flocculation test.

Seventeen patients (20.9 per cent) had a thymol turbidity of more than 4 units at this stage, with a mean for the entire group of 3.2 units, while fifty-nine (72.8 per cent) of the eighty-one patients had a more than 2 plus cephalin flocculation. Table I(B) shows the liver function tests of the seventy-one patients studied during the first forty-eight hours in the hospital. As can be seen, there was a slightly higher degree of abnormality when the tests were done during the acute phase shortly before or after specific therapy was begun.

Of the thirty patients whose liver functions were re-evaluated during convalescence, Table I(C), twenty-eight (93.3 per cent) showed a BSP retention of more than 4 per cent in 45 minutes on entry with a mean value of 18.4 per cent, whereas during convalescence only eight patients (26.6 per cent) showed abnormal BSP retention with a mean value of 4.7 per cent. The thymol turbidity in this group of thirty patients was abnormal in 30.0 per cent on admission, this figure dropping only slightly, to 23.3 per cent, during convalescence. The cephalin flocculation was determined to be 3 plus or more in

75 per cent of the group during the acute phase, and in 53.3 per cent of the group during convalescence. The return toward normal of the bromsulfalein and cephalin-cholesterol flocculation tests is clearly significant statistically when tested by the chi square test.

An attempt was made to correlate the incidence and degree of abnormal liver function with the height of the fever, the degree of leucocytosis, intensity of tachycardia and tachypnea, duration of illness before entry, presence of bacteremia, and age of the patients (Table II), as well as with the lobe or lobes involved (Table III). As can be seen by inspection of the data, there was no

TABLE II. CORRELATION BETWEEN VARIOUS PROGNOSTIC CRITERIA OF SEVERITY OF DISEASE AND DEGREE OF IMPAIRMENT OF LIVER FUNCTION

PROGNOSTIC CRITERION	LIVER FUNCTION TEST	LESSER SEVERITY*			GREATER SEVERITY†			T
		NUMBER	PER CENT ABNORMAL	MEAN	NUMBER	PER CENT ABNORMAL	MEAN	
Fever	BSP	38	89.5	14.1 ∓ 1.56	33	90.9	16.5 ∓ 1.92	0.07
	T. T.	38	19.5	3.0 ∓ 0.4	33	25.0	3.8 ∓ 6.5	0.04
	C. F.	38	74.5		33	75.0		
Leucocytosis	BSP	41	92.7	14.6 ∓ 1.65	30	86.8	15.2 ∓ 1.45	0.05
	T. T.	41	19.5	3.0 ∓ 0.5	30	27.3	3.2 ∓ 0.4	0.03
	C. F.	41	70.7		30	72.7		
Tachycardia	BSP	36	91.6	12.9 ∓ 1.95	35	88.6	14.9 ∓ 1.64	0.03
	T. T.	36	19.8	3.4 ∓ 0.5	35	23.0	3.1 ∓ 0.31	0.03
	C. F.	36	88.2		35	64.0		
Tachypnea	BSP	40	85.0	1.45 ∓ 1.81	31	89.5	13.7 ∓ 1.80	0.02
	T. T.	40	22.5	3.1 ∓ 0.49	31	22.6	3.3 ∓ 0.51	
	C. F.	40	80.0		31	67.8		
Duration	BSP	32	83.8	12.1 ∓ 1.41	39	84.6	13.2 ∓ 1.74	0.02
	T. T.	32	28.1	3.3 ∓ 0.61	39	23.1	3.1 ∓ 0.42	0.03
	C. F.	32	68.8		39	76.9		
Bacteremia	BSP	37	83.8	19.4 ∓ 1.43	12	100.0	15.2 ∓ 1.64	0.03
	T. T.	37	31.8	4.4 ∓ 0.56	12	25.0	3.1 ∓ 6.45	0.07
	C. F.	37	73.9		12	50.0		
Age	BSP	39	87.2	14.8 ∓ 2.68	32	84.4	14.1 ∓ 1.74	0.02
	T. T.	39	17.9	3.1 ∓ 0.39	32	28.1	3.5 ∓ 0.46	0.04
	C. F.	39	77.0		32	65.6		

*Lesser severity indicates in the case of fever a level below 103° c. in the case of leucocytosis a value below 20,000, with reference to tachycardia a level below 120, tachypnea a value below 30, duration less than three days, with reference to bacteremia, its absence, and with reference to age, below 40 years.

†Greater severity refers to values above the limits noted above and with reference to bacteremia, its presence.

significant correlation between the severity of the pneumonia as judged by each of these criteria on the one hand and the degree or incidence of impairment of liver function on the other. In an attempt to assess the role of alcoholism in contributing to the impairment of liver function (Table IV), the patients were divided into three groups: (1) those with an unequivocal and frank history of alcoholism, (2) those with an equally clear-cut and consistent denial of alcoholism, and (3) a less well-determined group in which the history of alcoholism was not adequately defined. While the trend seemed to be for the greatest impairment to occur in the alcoholic group and the least in those that denied alcoholism, with the indeterminate group about midway between the two, these differences are not statistically significant ($t = 0.23$). This difference, however is about eight times as significant as that of any of the other comparisons already noted. In a much larger group it is possible that the effect of alcohol might be

TABLE III. CORRELATION BETWEEN LOBE INVOLVEMENT AND LIVER FUNCTIONS IN 71 CASES

LOBE	LLL		LUL		RUL		RML		RLL		MULTIPLE	
NUMBER OF CASES	19		4		17		4		20		7	
	% ABNORMAL	MEAN	% ABNORMAL	MEAN	% ABNORMAL	MEAN	% ABNORMAL	MEAN	% ABNORMAL	MEAN	% ABNORMAL	MEAN
BSP	89.5	10.7	75	12.2	76.5	13.7	75	20.5	85	13.4	71.6	10.9
T. T.	21.1	4.1	25	4.7	17.6	2.4	50	3.9	20	3.0	28.3	3.0
C. F.	66.7	--	75	--	88.2	--	75	--	75	--	71.6	--

demonstrable. Since an accurate appraisal of the dietary background of these patients was not obtained, the role of diet cannot be distinguished from that of alcohol. Appraisal of the correlation between the infecting pneumococcus type and the degree or incidence of liver functional impairment showed too

TABLE IV. RELATIONSHIP BETWEEN A PREVIOUS HISTORY OF ALCOHOLISM AND IMPAIRMENT OF LIVER FUNCTIONS IN PNEUMONIA

	GROUP I, ADMITTED			GROUP II, DENIED			GROUP III, UNCERTAIN		
ALCOHOLISM	NUMBER	PER CENT ABNORMAL	MEAN	NUMBER	PER CENT ABNORMAL	MEAN	NUMBER	PER CENT ABNORMAL	MEAN
BSP	36	97.2	17.4 ∓ 1.56	15	86.6	10.5 ∓ 1.93	20	85.0	13.8 ∓ 2.65
T. T.	36	22.2	3.6 ∓ 0.54	15	13.3	2.6 ∓ 0.98	20	35.0	3.4 ∓ 0.39
C. F.	36	78.9	–	15	73.3	–	20	60.0	–

broad a distribution of types for valid statistical comparison. These revealed, however, no apparent consistent differences between the various types. Since the group consisted almost entirely of Negroes, the role of the race could not be evaluated.

Of the thirteen patients with pneumonia and jaundice studied (Table V), increased urine urobilinogen was noted in all. Bile was demonstrated in the urine of ten of the eleven patients in whom the search was made. Increased amounts of direct-reacting bilirubin were found in the blood of all seven of the patients in whom the test was done. Patients J. M. and G. J. showed bile in the urine when the icterus index was only 13 and 15 respectively. The findings of bile and increased urobilinogen in the urine and distinctly increased direct-reacting blood bilirubin in almost the entire group with jaundice indicate that the jaundice was hepatocellular in origin. Even more critical is the presence of bile in the urine with only slight elevation of the icterus index. It is significant that ten of the patients who developed jaundice gave a history of alcoholism. It should be noted from Table V that, by the criteria of severity previously discussed, this group of jaundiced patients was quite evenly divided.

DISCUSSION

When any of the known functions of the liver have been studied in pneumonia,[17-32] impairment to a significant degree in a large proportion of the patients has been noted (Table VI). Disturbance of the activity of the liver with respect to carbohydrate[18-20] and protein[21-27] metabolism, biliary pigment physi-

TABLE V. DATA ON 13 PATIENTS WITH PNEUMONIA AND JAUNDICE

PATIENT	SEX	AGE (YR.)	HISTORY OF ALCOHOLISM	LOBE	TYPE	BLOOD CULTURE	T.	P.	R.	W.B.C.	LI.	URINE BILE	VAN DEN BERGH	BILIRUBIN DIR.	BILIRUBIN INDIR.	BSP (%)	T. T.	C. F.
N. L.	F	40	Yes	R and LLL	N.D.	0	103°	110	28	9,450	25	Pos.	Dir. immed.	N.D.		28	6.2	4
R. B.	M	31	Yes	RUL	3	0	102°	110	30	33,000	15	N.D.	N.D.	N.D.		26	1.5	4
G. J.	M	36	Yes	LUL	0	N.D.	104°	128	36	17,000	15	Pos.	Dir. immed.	N.D.		12	2.8	4
J. M.	M	27	Yes	RUL	4	0	101°	120	28	14,000	13	Pos.	N.D.	N.D.		24	1.8	2
W. F.	M	39	Denied	RML	21	0	104°	106	22	29,000	60	Pos.	Dir. immed.	5.1	6.6	50	6.9	4
N. B.	M	40	Yes	RML	0	0	101°	100	24	9,450	40	Pos.	Dir. immed.	N.D.		42	2.4	3
E. T.	F	34	Yes	RLL	0	0	102°	112	20	26,000	15	Pos.	Dir. immed.	N.D.		24	4.5	4
R. A.	M	29	Yes	LLL	1	0	104°	120	32	9,100	85	Pos.	Dir. immed.	5.4	5.5	N.D.	3.1	4
W. P.	M	30	Yes	LLL	8	0	101°	100	40	22,500	15	Neg.	N.D.	N.D.		27	3.8	4
R. M.	M	34	Denied	LUL	0	0	103°	114	24	15,500	65	Pos.	Dir. immed.	N.D.		N.D.	N.D.	4
A. T.	M	39	Yes	RLL	14	14	104°	126	34	13,800	50	Pos.	N.D.	N.D.		32	5.4	4
E. P.	F	43	No data	RUL	N.D.	9	104°	112	28	36,500	18	N.D.	N.D.	N.D.		18	3.1	2
A. T.	M	39	Yes	RLL	14	14	101°	90	24	5,500	50	Pos.	N.D.	N.D.		28	4.1	4

N.D., Procedure not performed.
0, No organism demonstrated.

ology,[17, 19, 27-30] cholesterol formation,[32] and dye excretion[29, 31] has been noted. The incidence of abnormal bromsulfalein retention and a positive cephalin-cholesterol flocculation test observed in this study agrees well with the described incidence of other liver functional impairment. It is of interest that the thymol turbidity test was abnormal in this group much less frequently than the cephalin-cholesterol flocculation but, when abnormal, returned to normal more slowly than the latter.

It has been shown that hepatic impairment may result from fever[34, 35, 36] and anoxia;[28] it also has been suggested that toxemia could be responsible for the liver damage of pneumonia.[29] None of these factors, however, seemed to be of major importance in this series, since there was no correlation between the

TABLE VI. ABNORMALITIES OF LIVER FUNCTION OBSERVED IN PNEUMONIA

TYPE OF FUNCTION	PROCEDURE	RESULT	AUTHOR
Metabolism of bilirubin	van den Bergh	Direct	Elton[30]
		prompt biphasic	Zimmerman and Thomas (Table V)[36]
	Bilinuria	Positive	Zimmerman and Thomas
	Urobilinogenuria	Increases	Wilbur and Addis[17]
			Curphey and Solomon[19]
			Harris[29]
Ability of liver to excrete foreign substances	Tetrachlorphthalein	Impaired	Ottenberg, Rosenfeld, and Goldsmith[31]
	Bromsulfalein	Impaired	Harris[29]
			Zimmerman and Thomas (Table I)
Activity of the liver in carbohydrate metabolism	Galactose tolerance	Abnormal	Canali[18]
	Levulose tolerance	Abnormal	Curphey and Solomon[19]
	Glucose metabolism	Alimentary glycosuria	Pisani[20]
Activity of the liver in protein metabolism	Plasma protein level	Depressed	Vassel[22]
			Moen and Reimann[23]
	Albumin level	Elevated	Bing[26]
	Urea formation	Depressed	Cohen and Levin[21]
	Cephalin-cholesterol flocculation	Abnormal	Wade[27]
			Zimmerman and Thomas (Table I)
	Thymol turbidity	Abnormal	Zimmerman and Thomas (Table I)
Activity of the liver in lipid metabolism	Blood cholesterol	Depressed	Chierici[32]

intensity or frequency of liver impairment and (1) the height of the fever, (2) the degree of anoxemia as judged by multiple versus single lobe involvement or the extent of tachypnea, and (3) the severity of the pneumonia as judged by the usual criteria including age of the patient. This is in agreement with the observations of King and Leslie[37] and Harris.[29] Furthermore, as is implicit in the evidence of Turner and associates[38-40] of the increased incidence of jaundice in pneumonia in dogs on deficient diets, the previous status of the liver conditions the intensity of liver damage with pneumonia. While the assessment of the role of alcoholism in the present group is difficult, the results show a trend in keeping with these conclusions.

In spite of the lack of correlation with any of the factors mentioned, it is possible that all contribute to the liver damage, which is, however, modified by the previous nutritional status of the patient. It is possible that the "alarm

reaction'' shown to occur in pneumonia and other infections[43] is the mechanism for the occurrence of the liver damage. Other clinical entities considered to represent the alarm reaction are frequently associated with impairment of liver function and at times hepatocellular jaundice.[44-46] That the alarm reaction occurs in acute infections is corroborated by the eosinopenia[47] and increased urinary corticoid excretion[48] described, but not enough data have been accumulated to adequately compare the intensity of the change in pneumonia with that in other infections.

Jaundice in Pneumonia.—In the standard medical textbooks[49-56] the problem of jaundice in pneumonia receives scant reference. While the monographs on pneumonia[54, 55] comment on the occurrence of jaundice and list the possible causes as (1) hemolytic, (2) hepatocellular, and (3) obstructive, and while the extensive literature on liver function and jaundice in pneumonia has been extensively reviewed,[57] all of these authoritative sources fail to critically choose the most reasonable of these three possibilities of the genesis of the jaundice.

The extensive evidence in the literature bearing on the nature of the jaundice in pneumonia has been summarized in Table VII. The concept of an obstructive type of jaundice is the oldest theory, that of a hemolytic origin is the most popular, while the recognition of a hepatocellular origin has received only brief attention.

It is clear that the thesis of a purely hemolytic origin of the jaundice in pneumonia fails to explain or take into account (1) the presence of direct-reacting bilirubin in the serum and of bile in the urine, (2) the disturbance of the entire spectrum of liver function in pneumonia as discussed, and (3) the demonstration by Turner[38-40] and his group of the increased incidence of jaundice in the presence of nutritional liver damage. The theory of obstruction of the biliary tree is not tenable because (1) it is not borne out by most histologic studies, (2) it fails to explain the high incidence of impairment of liver function, and (3) it is not compatible with the persistently observed urobilinogenuria. That the jaundice is largely or entirely hepatocellular is confirmed by (1) the invariable presence of hepatic cell lesions in fatal cases,[28, 64] (2) the almost invariable impairment of all modalities of liver function, and (3) the presence of increased amounts of direct-reacting bilirubin in the blood and, concomitantly, bile and urobilinogen in the urine. Only a hepatocellular origin of the jaundice can explain all of the observed phenomena. To invoke hemolysis as a complementary factor in the production of the jaundice is only restating a fact that Watson[68] has emphasized. The degree of jaundice, whether hepatocellular or obstructive in origin, depends on the rate of blood destruction. Therefore, in both types of jaundice, when the rate of blood destruction is reduced, icterus is less marked, and when blood destruction is accelerated, the converse is true. It would seem, therefore, that the jaundice in pneumonia is an extension of the liver damage which almost invariably accompanies the disease, and as in any other hepatocellular jaundice, the degree of icterus must be dependent upon the rate of hemoglobin breakdown and turnover.

TABLE VII. THEORIES OF GENESIS OF JAUNDICE IN PNEUMONIA

THEORY	EVIDENCE	SOURCE	CONTRARY EVIDENCE
Obstructive			
1. Large bile ducts or duodenum	1. Anatomical changes described in ducts and duodenum	1. Abramow[3] Eppinger[60]	1. and 2. not confirmed by Klemperer[64] and others[42]
2. Canaliculi	2. Cholangiolitis described	2. Gerbst[66]	
3. Splinting of right diaphragm	3. A greater incidence of jaundice described formerly with right lower lobe pneumonia	3. French[51]	3. Greater incidence of jaundice with right lower lobe pneumonia not noted in present study (Table V)
Hepatocellular	Impairment of all modalities of liver function	Sources summarized in Table VI	None
	Constant changes in liver cell in fatal cases of pneumonia	Rich[28] Blake and Cecil[67]	None
	Presence of bile and urobilinogen in urine concomitantly	Elton[30] Bittorf[6] Zimmerman and Thomas (Table V and text)	
	More frequent in human beings or dogs with malnutrition, i.e. previous liver damage	Turner and co-workers[38-40] Himsworth[58]	None
Hemolytic (Bilirubin over-production)	Bilirubin or blood intratracheally causes increased blood bilirubin	Maugeri[63]	Turner and co-workers attempted to confirm the work of Maugeri and failed
	Increased red blood cell fragility	Bittorf[6]	Chance findings not reported elsewhere and not generally true
	Rapidly developing anemia in 2 cases	Bittorf[6]	
	Siderosis of lung	Eppinger[60]	Not confirmed by other pathologists[58, 59]
	Bilirubin in rusty sputum	Chierici[32]	None. Based, however, on color of sputum, not on chemical analysis, and bilirubin even if present in sputum does not indicate that such bilirubin is responsible for or contributes to blood icterus
	Indirect van den Bergh always	Chierici[32]	Not true. When jaundice is present, the van den Bergh is almost always direct (Elton[30]), Zimmerman and Thomas (Table V)
	Lack of itching bradycardia, and acholic stools	Chierici[32]	Does not exclude hepatocellular jaundice, for the incidence of these findings in the classical varieties of hepatocellular jaundice is extremely variable

The explanation offered by Rich[28] for the occurrence of jaundice in pneumonia is not too dissimilar from that presented here. He emphasized the frequent impairment of liver function and suggested that the damaged liver was unable to accept the increased amounts of bilirubin returning to it. This mechanism, he indicated, would operate in any case of hemolytic jaundice, but

becomes exaggerated by the added impairment of liver function incident to the anoxia of pneumonia. The concept of Rich differs from the one presented here only in that he felt that "regurgitation" of bile with the consequent presence of bile in the urine was rare in pneumonia, while in the present study bile was observed in the urine in almost all of the jaundiced patients, including the minimally jaundiced ones.

Implications of Liver Dysfunction in Pneumonia.—In view of the extremely low mortality of patients with pneumonia since the advent of antibiotic therapy, no correlation between the degree of impairment of liver function and prognosis could be established in the present study. There were only two deaths in the group, one in a jaundiced patient, the other in a nonjaundiced patient. The lack of correlation, however, between the severity of the pneumonia as judged by the usual criteria and the intensity of impairment of liver function as noted in this series and reported by others[19, 29] suggests that a greater intensity of impairment of liver function does not prejudice the outcome. It was noted before the days of antibiotic therapy that persistence of impairment of liver function was seen more frequently in patients who succumbed to the disease than in those who survived.[19]

In the older literature[1, 2] and in one recent study,[37] the opinion was held that pneumonia with jaundice is associated with a poorer prognosis than pneumonia without this complication. Most observers, however, feel that the presence of jaundice does not impair the prognosis.[47, 69, 70, 71] Indeed one author has suggested that jaundice in a certain highly specific range actually improves the prognosis.[30] In general it may be said that the prognosis in jaundiced patients must be evaluated by the same criteria as in those patients with pneumonia who are not jaundiced.

It should be noted that, as in many other systemic diseases,[36] pneumonia is an example of "nonspecific" impairment of liver function, illustrating the need for further study of the factors that diminish liver function, and emphasizing the fact that impaired liver function and hepatocellular jaundice do not necessarily mean intrinsic liver disease.

Furthermore, in the light of the published data and of the present study, the jaundice of pneumonia is not to be regarded as an occasional peculiar phenomenon but rather as an extension of a frequently occurring process, namely, impairment of liver function.

SUMMARY AND CONCLUSIONS

From a review of the literature on liver function and jaundice in pneumonia and from a study of ninety-four patients with pneumonia, thirteen of whom had jaundice, the following conclusions were reached.

(1) Liver function is widely and almost invariably impaired in pneumonia.

(2) The impairment of liver function cannot be directly correlated with the intensity of the infection as measured by the usual criteria.

(3) In the absence of other correlative factors, it is suggested that the degree of impairment may be associated with the degree of anoxia, fever, and

intensity of the infection, conditioned by previous liver status, and mediated through the alarm reaction which is said to occur in pneumonia.

(4) Jaundice in pneumonia is characterized by the occurrence concomitantly of bile and increased urobilinogen in the urine, and is, therefore, hepatocellular in type. It cannot be regarded as an isolated phenomenon, but is rather a manifestation of the impairment of hepatocellular function which frequently accompanies the pneumonic process.

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STUDIES ON EXPERIMENTAL DIABETES MELLITUS, AS PRODUCED BY ORGANIC REAGENTS

Oxine Diabetes and Dithizone Diabetes

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IN RECENT years studies on experimental diabetes mellitus have made remarkable progress. Since von Mering and Minkowski[28] first discovered diabetes by totally extirpating the pancreas, three other types of experimental diabetes have been reported to date. Evans, Meyer, Simpson, and Reichert,[13] Baumann and Marine,[5] Houssay, Biasotti, and Rietti[20]—these three groups of investigators reported in 1931-1932 the production of experimental diabetes by injecting pituitary anterior extracts. Pathologic changes in the islets in pituitary diabetes were studied by Richardson and Young[35, 36] and Ham and Haist.[18] According to them, lesions in the islets were observed only in beta cells and were identified essentially with that of partially pancreatectomized diabetes. Progressive loss of granules in beta cells and hydropic degeneration were found. In persistent diabetes the islets were reduced in size with disappearance of beta cells and were composed chiefly of alpha cells and a few agranular or hydropic cells.

Since alloxan diabetes was discovered in 1943, numerous researches[10] have been performed. Dunn, Sheehan, and McLetchie[11] first discovered a selective necrosis of the islets following intravenous injection of alloxan into rabbits. Bailey and Bailey[1] could produce alloxan diabetes by preventing hypoglycemia after repeated intravenous administration of dextrose. As for the histologic changes of the islets, Bailey, Bailey, and Hagan[3] reported that degenerative changes were already apparent in the initial hyperglycemic stages and in the hypoglycemic stages they progressed further, approaching those of the diabetic phases. They considered that histologic lesions in different phases of blood sugar changes formed one continuous process. Finally, Dohan and Lukens[9] (1948) observed a persistent hyperglycemia in a normal cat and two half-pancreatectomized ones after the cessation of the daily injection of dextrose.

About the diabetogenic actions of other various chemical substances than alloxan, in 1937 Jacobs[23] noted that a variety of chemicals related to alloxan—alloxanic acid, dialuric acid, isodialuric acid, barbituric acid, isobarbituric acid, alloxantin, murexide, mesoxalic acid, parabanic acid, oxaluric acid, formyloxaluric acid, or formylurea—had no effect on blood sugar. Kennedy nd Lukens[24] found no diabetogenic action in nitrates, methylene blue, and sodium tartrate. In 1943 Dunn and co-workers[11] mentioned that a quinoline compound, styrylquinoline No. 90, had such effects as those made by alloxan, but oxalic acid, uranium, guanidine, and uric acid had no effect. In

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1944 Thorogood[40] reported that parabanic acid, uramil, violuric acid, benzoylbarbituric acid, and thiouracil had no diabetogenic action like alloxan, and in the same year Goldner and Gomori[15] tested in animals dialuric acid, alloxantin, violuric acid, barbituric acid, N-dodecylbarbituric acid, and the oxidizing substances, sodium nitroferricyanide, sodium molybdate, ceric acid, quinine, and persulfate, but found no effects similar to those made by alloxan. They also failed in experiments with two quinoline compounds, quinoline and cinchophen and concluded that the oxidizing effect of alloxan might not be important in the production of diabetes.

Bailey, Bailey, and Leech[2a] demonstrated that ninhydrin, alloxan-6-phenylhydrazone-*p*-sodium sulfonate, cytosine, isocytosine, glyoxal, and styrylquinoline besides the above-mentioned chemicals had no diabetogenic action. Weinglass and Tagnon[41] reported that no effect on blood sugar was observed after intravenous injection of chymotrypsin which makes insulin inactive in vitro.

Koref, Vargos, Rodriguez, and Telchi[25] reported the transitory hyperglycemia and typical diabetes caused by intravenous injection of alloxantin. Brückmann and Wertheimer[7] showed that styrylquinoline No. 90 had no diabetogenic action but that diabetes could be produced by methyl-alloxan, dimethylalloxan, dialuric acid, and methyldialuric acid. Bailey, Bailey, and Leech[2b] confirmed the diabetogenic action of dialuric acid. Recently, Griffiths[17] discovered that the intraperitoneal injection of uric acid into rabbits, fed with the methionine- and cystine-deficient diet, caused permanent diabetes like alloxan.

As mentioned, numerous researches about the production of diabetes by chemicals related to alloxan and others have been reported to date since the discovery of alloxan diabetes. However, the essential points of alloxan diabetes remain still obscure. It may be concluded that remarkable progress has not yet been made regarding the fundamental mechanisms involved.

THEORETICAL BASIS OF EXPERIMENTAL DIABETES PRODUCED BY ORGANIC REAGENTS

In 1942, Okamoto[32, 33] reported the histochemical method of detecting zinc in tissues by diphenylthiocarbazide and dithizone. Applying this method, he discovered that zinc could be detected distinctly in the islets of Langerhands in a large part of vertebrate animals. He reported that zinc in the islet cells increased in a starved rabbit which lost about 30 per cent of its body weight. Zinc, however, disappeared when it was fed with a carbohydrate diet, but not conspicuously when fed with a protein or fat diet. From these facts, Okamoto assumed that zinc detected in the islets of Langerhans might play a role in carbohydrate metabolism.

Furthermore, Scott[38] (1934) reported that crystalline insulin contained zinc, and then Scott and Fisher[39] reported that this zinc might connect with insulin chemically and that the action of insulin was delayed when zinc was added to insulin.

Since chemical analysis[14] shows that a moderate amount of zinc is contained in the pancreas, it may be conceived that zinc in the islets may connect with insulin and have an important meaning.

On the other hand, Denigès[8] reported that alloxan could react with zinc, iron, and other metals.

From these facts, the author and Okamoto presumed that the mechanism by which alloxan injures the islets selectively might be based on alloxan in the blood stream connecting

with the zinc in the islets and injuring their cells, and that diabetes might be produced by organic reagents other than alloxan which react with zinc. It was on these considerations that the present studies were undertaken.

METHOD AND MATERIALS

Organic reagents which react with zinc are numerous and it is necessary to select reagents which comply with the following conditions. They must be: (a) able to make a solution compatible with blood or with body fluid, (b) able to react at body temperature and at the pH of tissue, (c) able to react in a prompt, specific, and sensitive manner, (d) able to effect to some extent an injurious action upon the cells.

The following six reagents were selected for experiment: oxine (8-hydroxyquinoline), dithizone (diphenylthiocarbazone), quinaldinic acid, anthranilic acid, 4-hydroxybenzthiazol, and diphenylthiocarbazide. Chemical natures of these reagents are shown in Table I.

As oxine is insoluble in water, its hydrochloric salt was used as 1 or 2 per cent solution of oxine in dilute hydrochloric acid; dithizone as solution in dilute ammoniacal solution; anthranilic acid as 5 to 10 per cent solution in distilled water; and quinaldinic acid as 3 per cent solution in distilled water. 4-Hydroxybenzthiazol as a solution in dilute hydrochloric acid similar to oxine and diphenylthiocarbazide as saturated solution in 50 per cent alcohol were employed. Toxic doses of these reagents are shown in Table I.

For experimental animals adult rabbits were employed in this study. Blood sugars were determined by the Hagedorn-Jensen method and urine sugars were examined by the Benedict qualitative method. Immediately after the animals were sacrificed or died from hypoglycemic convulsions, autopsies were performed at various intervals as indicated in the results. The pancreas was first removed and fixed in Bouin's fluid, and then the other organs were fixed in Formalin solution. For the staining of granules in the islets of the pancreas, the chromium hematoxylin phloxin method adopted by Gomori[16] was used. The other tissues were examined by means of the routine hematoxylin-eosin staining.

For the histochemical staining of zinc, materials were fixed in absolute alcohol and embedded in paraffin, and the diphenylthiocarbazide method of Okamoto[32] was employed, the outline of which is as follows:

(1) Rinse section for two to three hours or more at room temperature in a mixed solution of

Ammoniac solution of pH 8.4 to 9.0	50 c.c.
Saturated solution of diphenylthiocarbazide in 60 per cent alcohol (two to four days after preparation)	1 to 3 c.c.

(2) Wash in water.

(3) Stain nucleus with hematoxylin, wash in water, and mount in glycerin. By this method the existence of zinc in tissues is proved by deep red color.

RESULTS

Changes of Blood Sugar by Oxine and Occurrence of Oxine Diabetes.—Intravenous injection of a small dose of oxine (10 to 20 mg. per kilogram) caused only transitory hyperglycemia which returned to normal value after twenty-four hours. However, following the intravenous injection of 50 mg. per kilogram oxine, one could observe the three phases in blood sugar changes: an initial hyperglycemia, then hypoglycemia, and then permanent hyperglycemia. Initial fluctuation of blood sugar after intravenous injection of 50 mg. per kilogram oxine is shown in Table II and Fig. 1.

Most of the rabbits showed initial hyperglycemia in one to four hours after injection of oxine, and then in ten to twenty-four hours prominent hypoglycemia was observed. Hypoglycemic convulsions and death were observed

TABLE I. CHEMICAL NATURE, ESPECIALLY REACTIONS WITH ZINC, OF THE ORGANIC REAGENTS USED IN THIS EXPERIMENT

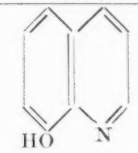
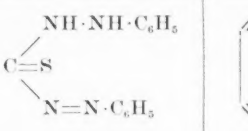
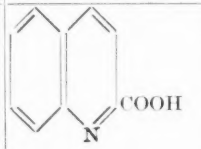
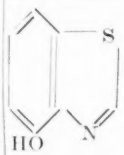
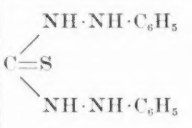
	OXINE (8-HYDROXYQUINOLINE)	DITHIZONE (DIPHENYLTHIOCARBAZONE)	ANTHRANILIC ACID	QUINALDINIC ACID	4-HYDROXYBENZTHIAZOL	DIPHENYLTHIOCARBAZIDE
Constitutional formula						
Nature	Colorless or slight yellow needle crystal, melts at 75° C., soluble in organic solvents	Black or black-brown pulver, insoluble in water a. dilute acid, soluble in alcohol, chloroform, carbontetrachloride a. ammoniac	White pulver, melts at 145° C., alkali salt soluble in water	Needle crystal, melts at 155-160° C., soluble in hot water a. in alkaline solution	White or slight yellow crystal, melts at 143° C., insoluble in water, soluble in hydrochloric acid	Gray-red crystal, soluble in alcohol
Reaction with zinc	Green precipitation	Red color reaction	White precipitation	White precipitation	White precipitation	Red precipitation
Moderate pH and sensitivity in reaction with zinc	pH 4.7-13.33 1:1,000,000	Neutral 1:1,000,000	Neutral or weak acid 1:2,000,000	Neutral or weak acid 1:1,000,000		Weak alkali
Metals which react with these reagents	Cu, Ag, Au, Be, Mg, Ca, Zn, Sr, Cd, Ba, Hg(I, II), Al, Sn, Fe (II, III), Co, Ni	Pb, Au, Ag, Hg, Cu, Sn (II), T (I), Bi, Zn, Co, Ni, Cd	Zn, Co, Ni, Cd, Cu, Pb, Hg (II)	Cu, Cd, Zn, U, P, As, Pb, Ni, Co	Zn, Ni, Co	Pb, Bi, Co, Cu, Cd, Ni, Zn, Al, Ca, Ba, As, Sb, Ag, Au, U, Cr, V, Hg, Th, Ce, Mo
Lethal doses for mouse (intravenous)	0.05 mg./Gm.	?	1 mg./Gm.	0.09 mg./Gm.	0.05 mg./Gm.	?
Lethal doses for rabbit (intravenous)	65 mg./kg.	?	1,400 mg./kg.	Over 100 mg./kg.	Circa 70 mg./kg.	?
Literature	Berg,[6] Mellan,[27] van Nieuwenburg,[31] Naito,[30] Kuroda[26]	Mellan,[27] van Nieuwenburg,[31] Naito,[30] Kuroda[26]	Mellan,[27] van Nieuwenburg,[31] Naito,[30] Kuroda[26]	Mellan,[27] van Nieuwenburg,[31] Naito,[30], Kuroda[26]	Erlenmyer,[12] Sakaguchi[37]	Parri, [34] van Nieuwenburg[31]

TABLE II. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF OXINE AND PROCESS OF OXINE DAABETES IN RABBITS

RABBIT	SEX	INITIAL BODY WEIGHT (GM.)	BLOOD SUGAR (MG. %)											DURATION OF DIABETES (DAYS)	AVERAGE BLOOD SUGAR (MG. %)
			BEFORE INJECTION	AFTER											
				1 HR.	2 HR.	4 HR.	8 HR.	12 HR.	16 HR.	18 HR.	22 HR.	24 HR.	29 HR.	48 HR.		
3	♂	2,180	123	183		212				49	64		76	261	15	233
5	♀	2,120	108	136	63	63	34			(died)		29				
8	♂	2,145	117	192	217	123		144		66	(died)					
9	♂	2,080	94	149	214	215		52		(convulsion)				289	9	290
10	♂	2,120	108	190	183	73		61			(died)		40			
11	♀	2,290	98	164	194	229		208		120		27	(died)			
13	♂	2,030	124		316	338				(convulsion)			278		3	270
17	♂	2,230	90		96	71			71			74		350	90	356
18	♀	2,600	92		102	88			112			137		360	120	370

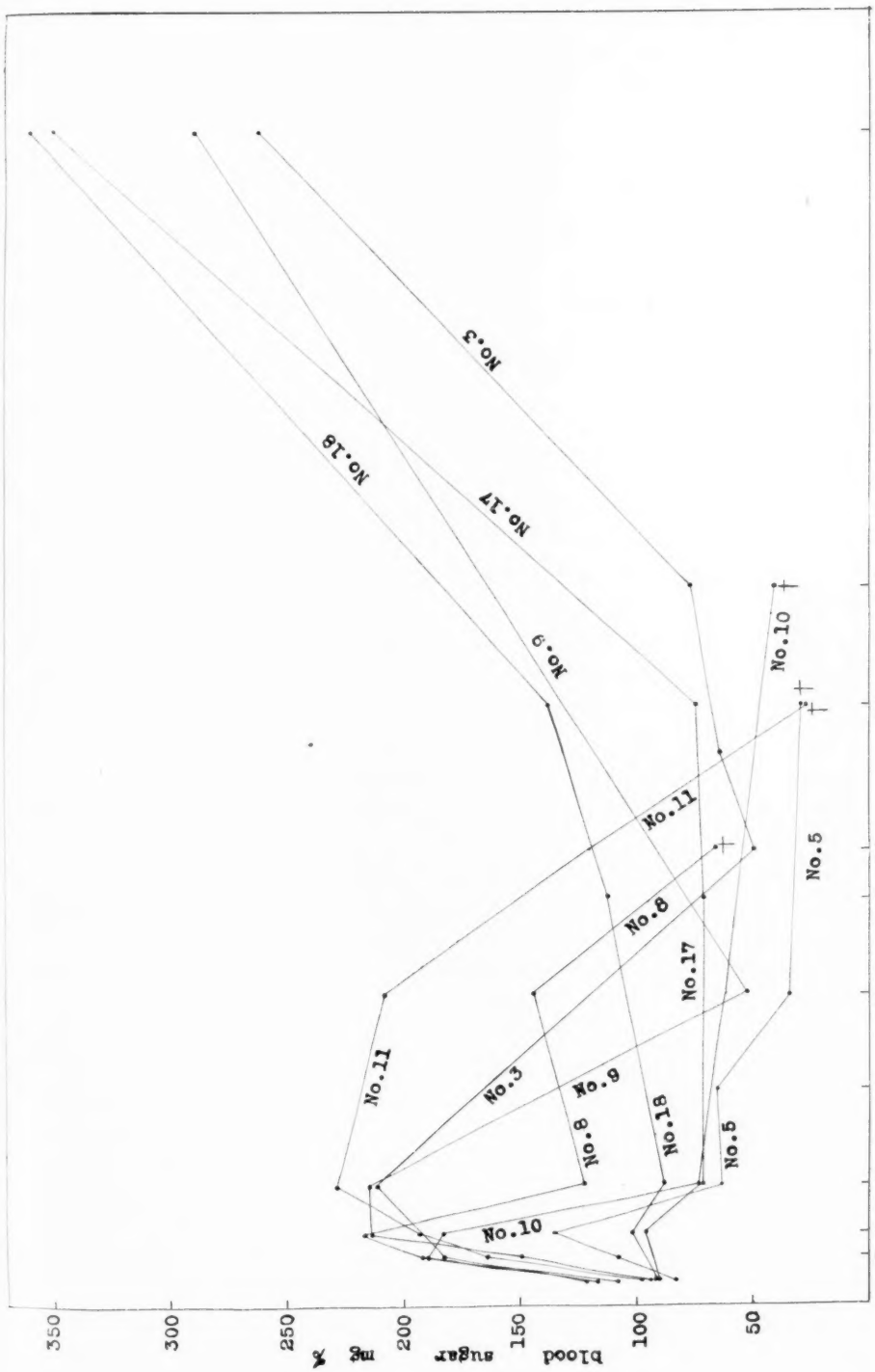


Fig. 1.—Changes of blood sugars during forty-eight hours after intravenous injection of 50 mg. per kilogram oxine in rabbits.

occasionally in severe cases, but most of the animals recovered without treatment or by the administration of 20 to 30 Gm. glucose. Then blood sugars increased gradually and finally continuous hyperglycemia was observed after forty-eight hours. By injecting the same dose of oxine, a few rabbits exhibited hyperglycemia which reached its peak in two to four hours and then returned to normal value after twenty-four to forty-eight hours. Occurrence of oxine diabetes is shown in Table III.

TABLE III. PRODUCTION OF OXINE DIABETES IN RABBITS

DOSES OF OXINE INJECTED INTO EAR VEIN (MG./KG.)	TOTAL NUMBER OF RABBITS	NUMBER OF RABBITS WITH PERMANENT DIABETES	NOTE
10	2	0	Temporary diabetes
20	3	0	Temporary diabetes
50-60	14	10	4 rabbits temporary diabetes 4 rabbits hypoglycemic death

Rabbit 18 sustained diabetes with 260 to 552 mg. per cent blood sugar for 120 days with appearance of lipemia, loss of subcutaneous fat, and slight excretion of aceton bodies in the urine, and then was sacrificed for histologic examination. In general, after the injection of diabetogenic doses of oxine, rabbits fall immediately and salivate. Nerosis of local tissue as the result of thrombosis of the ear vein is frequently observed.

Changes of Blood Sugar by Dithizone and Occurrence of Dithizone Diabetes.—A red color solution of dithizone, produced by dissolving 30 to 200 mg. per kilogram dithizone in 0.2 to 0.5 per cent ammoniac solution, and warmed to 60° to 70° C. for ten minutes, was injected intravenously into the rabbits. Changes of blood sugars are as listed in Table IV and Fig. 2.

Immediately after injection, sudden initial hyperglycemia was observed after one to four hours, and then a hypoglycemic phase was noted after eight to twelve hours, frequently with severe hypoglycemic convulsions. If animals recovered from the hypoglycemic phase, occurrence of permanent diabetes was observed after twenty-four hours.

Fluctuation of blood sugars was somewhat variable in each animal. Few animals showed hypoglycemia immediately without initial hyperglycemia, and a few sustained hypoglycemia even after twenty-four hours. Most of the animals showed permanent hyperglycemia after twenty-four hours, but a few only after forty-eight hours. Occurrence of dithizone diabetes is shown in Table V.

Rabbits which showed remarkable hypoglycemia had a tendency to develop severe diabetes. Most of the rabbits showed conspicuous local edema at the site of injection, and occasionally in other parts, but without other remarkable general symptoms. This local damage might be caused by the ammoniac solution. As the urine showed yellow-orange or red color after injection, it may be presumed that dithizone or its derivatives might be excreted within

TABLE IV. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF DITHIZONE AND PROCESS OF DITHIZONE DIABETES IN RABBITS

RABBIT	SEX	INITIAL BODY WEIGHT (GM.)	INJECTED DOSE OF DITHIZONE	BLOOD SUGAR (MG. %) BEFORE INJECTION	AFTER 1 HR.	2 HR.	4 HR.	8 HR.	12 HR.	18 HR.	24 HR.	48 HR.	DURATION OF DIABETES	AVERAGE BLOOD SUGAR (MG. %)
46	♀	2,330	50 mg./kg. D 0.5% A 20 c.c.	142	289	352	343	261			307	376	8 days	352
41	♀	3,200	50 mg./kg. D 0.5% A 32 c.c.	129	205	349	(died)							
48	♂	2,300	50 mg./kg. D 0.5% A 20 c.c.	117	237	314	297	216	133		158	279	Sacrificed after 48 hr.	
51	♂	2,500	100 mg./kg. D 0.2% A 20 c.c.	103	241	257		265			311	256	19 days	250
55	♂	2,250	200 mg./kg. D 0.5% A 20 c.c.	102	221	350	228	154	129		210	350	127 days	355
58	♀	2,850	200 mg./kg. D 0.5% A 20 c.c.	116	140	273	261	271	75		292		Sacrificed after 24 hr.	
59	♀	2,820	200 mg./kg. D 0.5% A 20 c.c.	132	56	50	14	75	(died)					
60	♂	2,880	200 mg./kg. D 0.2% A 60 c.c.	128	360	131	84				313	319	8 days	436
47	♀	2,760	50 mg./kg. D 0.5% A 20 c.c.	337*	178	312	310	350			279	300	85 days	293
62	♂	2,100	100 mg./kg. D 0.2% A 10 c.c.	101			166			89	(died)			
64	♂	2,200	100 mg./kg. D 0.2% A 10 c.c.	139		337	294			36	(died)			
65	♂	2,000	100 mg./kg. D 0.2% A 10 c.c.	141		281	124				59	296	43 days	294

D, dithizone; A, ammoniac solution.

*Permanent hyperglycemic state by previously injected dithizone.

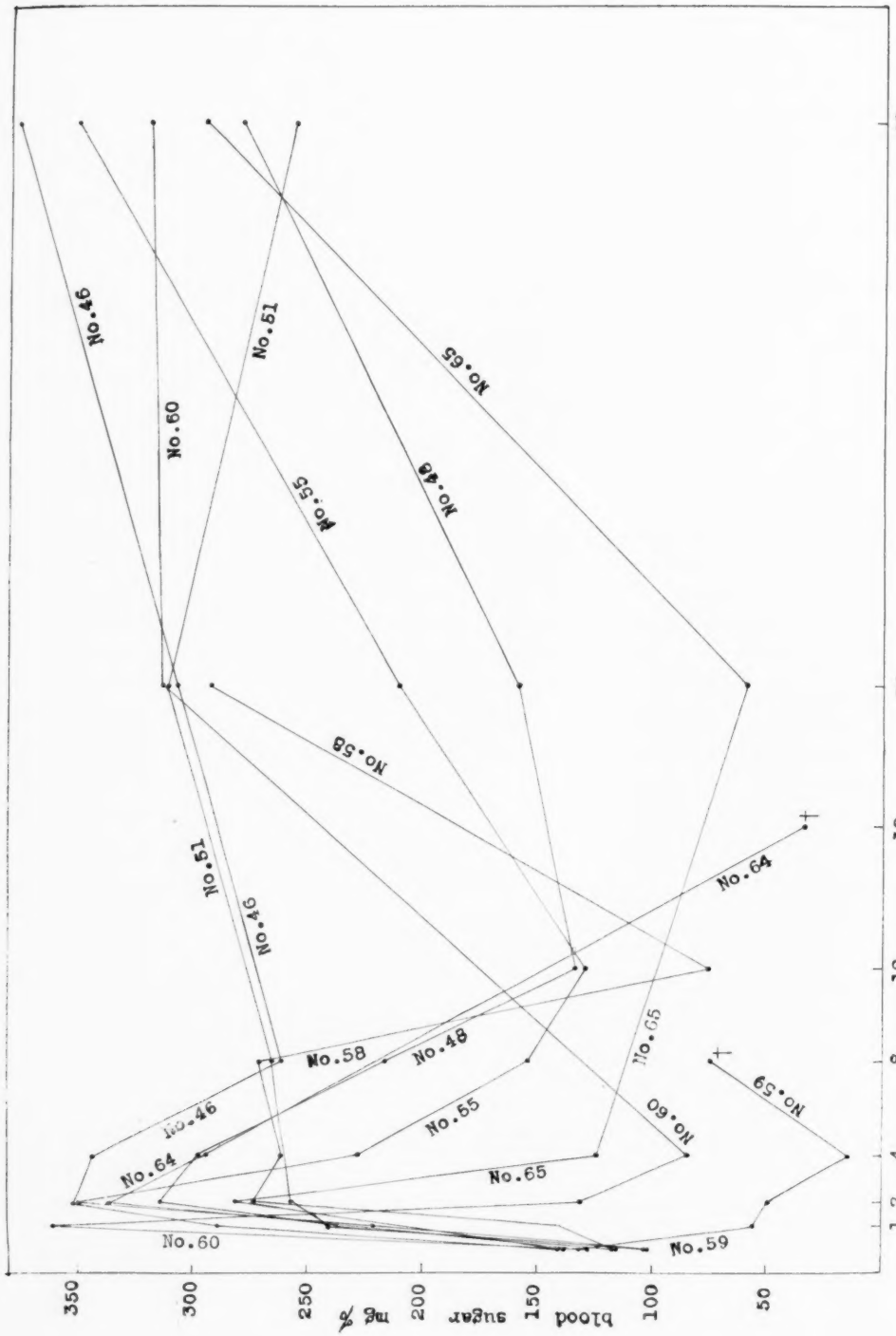


Fig. 2.—Changes of blood sugars during forty-eight hours after intravenous injection of 50 to 200 mg. per kilogram dithizone in rabbits.

TABLE V. PRODUCTION OF DITHIZONE DIABETES IN RABBITS

DOSES OF INJECTED REAGENT	TOTAL NUMBER OF RABBITS	NUMBER OF RABBITS WITH PERMANENT DIABETES	NOTE
D—30 mg./kg. 0.2% A—ca. 10 c.c./kg. warmed at 70° C. for 10 min.	3	1	2 rabbits temporary diabetes
D—50 mg./kg. 0.2-0.5% A—ca. 10 c.c./kg. warmed at 70° C. for 10 min.	2	2	
D—100 mg./kg. 0.2-0.5% A—ca. 10 c.c./kg. warmed at 70° C. for 10 min.	6	6	4 rabbits hypoglycemic death
D—200 mg./kg. 0.2-0.5% A—ca. 10 c.c./kg. warmed at 70° C. for 10 min.	7	7	2 rabbits hypoglycemic death

D, dithizone; A, ammoniac solution.

about twenty-four hours. When 500 mg. per kilogram dithizone was orally administered, initial hyperglycemia followed by fall of blood sugars to normal value was observed and finally hyperglycemia continued for three days.

Changes of Blood Sugar by the Other Zinc Reagents.—Quinaldinic acid, anthranilic acid, and 4-hydroxybenzthiazol caused no fluctuation of blood sugars by intravenous injection. Diphenylthiocarbazide caused no remarkable changes, except only slight transitory hyperglycemia in a few rabbits.

Histologic Changes of the Pancreas in Oxine Diabetes.—In all rabbits conspicuous changes were observed in the islets of Langerhans. In six hours after the intravenous injection of oxine, slight degenerative changes, pyknosis of the nuclei, disappearance of granules, and a few fine vacuoles in the cytoplasm were observed. In the rabbit sacrificed twenty-four hours after injection, there were degenerative changes and necros is of beta cells. Most of the nuclei were stained slightly and showed atrophy and necrosis; the cytoplasm appeared netlike or cloudlike. There remained only nuclei surrounded by fine vacuoles or hydropic spaces. Some cytoplasm appeared homogenous and the boundary of each cell was not clear. Alpha cells, however, remained normal in number and in the appearance of the nuclei and cytoplasm.

In three rabbits which died in eighteen to thirty-five hours with severe hypoglycemic convulsions, the most severe necrosis and disintegration of beta cells were observed. While alpha cells remained apparently normal at the periphery of the islets, cells which might be suggested as beta cells in the centers of the islets turned into homogenous or cloudy necrotic masses, stained slightly violet-red. The boundary of the cells became unclear and some cells contained vacuoles in the cytoplasm. In some islets, necrotic nuclei stained slightly red, or pyknotic nuclei or spaces corresponding to the nuclei, were observed grouped or scattered in the necrotic masses.

In some relatively slightly damaged islets, beta cells in the centers of the islets appeared sporadically in their normal appearance. Nuclei were pyknotic or necrotic, stained reddish, and degranulation, homogenization and many fine vacuoles were observed in the cytoplasm. Occasionally fragments of the

Fig. 2.—Changes of blood sugars during forty-eight hours after intravenous injection of 50 to 200 mg. per kilogram dithizone in rabbits.

destructed cells were seen scattered. The necrosis and disintegration of beta cells were most conspicuous in Rabbits 8 and 10 (Figs. 3, 4, and 5) and relatively slighter in Rabbit 11.

These changes were observed in similar degree in the whole pancreas, but a few islets remained relatively undamaged with the structure of their cells preserved. In these islets alpha cells remained normal in number, arrangement, and appearance of nuclei and cytoplasm, except that a few nuclei were pyknotic.

In the rabbit sacrificed forty-eight hours after injection, while alpha cells remained apparently intact, beta cells appeared apparently normal in number and character. However, they showed cloudiness with disappearance of granules and became homogenous with fine vacuoles, but preserved their boundaries indicating slight degenerative changes of beta cells.

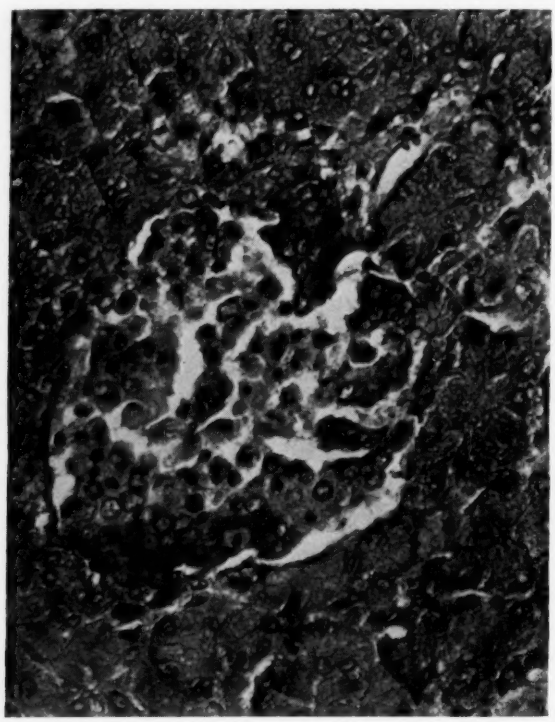


Fig. 3.—Islet of Langerhans of Rabbit 10 which died from hypoglycemia thirty-five hours after intravenous injection of oxine. Relatively slightly damaged islet. While alpha cells appear normal, beta cells in center of islet show pyknosis of nuclei and slight degenerative changes.

At seventy-two hours after injection the islets were 80 to 90 per cent composed of alpha cells. The beta cells decreased and appeared more packed with pyknotic nuclei, and partially their degenerated cytoplasm coalesced, becoming homogenous masses stained slightly red. In Rabbit 13 which died three days after injection of oxine from weakness due to persisting hyperglycemia, the islets were reduced in number and size and were composed

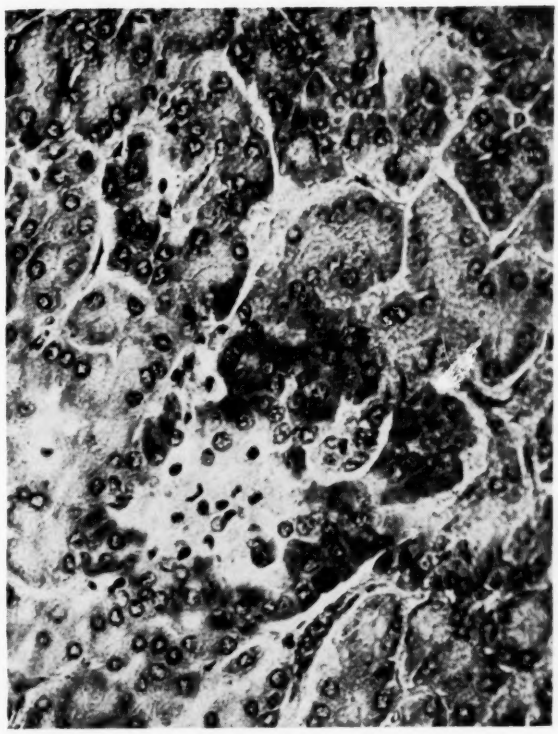


Fig. 4.—Islet of Langerhans of Rabbit 10 which died from hypoglycemia thirty-five hours after intravenous injection of oxine. Although peripherally arranged alpha cells show no change, beta cells in center of islet show moderate necrosis, still preserving its structure, and a few pyknotic nuclei. (Gomori's chromium hematoxylin phloxin stain.)

Fig. 5.—Islet of Langerhans of Rabbit 10 which died from hyopglycemia thirty-five hours after intravenous injection of oxine. Most severe necrosis and disintegration of beta cells are observed. Center of islet shows remarkable necrosis, forming homogenous mass, in which a few pyknotic nuclei remain. Alpha cells, however, remain apparently normal at periphery of islet.

chiefly of alpha cells. In other parts than alpha cells there were open spaces in which cloudy substances were stained slight violet and which might be regarded as beta cell remnants. Further, a few scattered deeply stained nuclei were observed there. The small islets were composed of two or three alpha cells.

In other specimens, ten days after occurrence of oxine diabetes, the number of beta cells remained normal, but nuclei became granular with a few chromatin substances. Their cytoplasm contained numerous fine vacuoles and appeared transparent and netlike, but alpha cells remained normal.

In the rabbit sacrificed after 120 days of oxine diabetes, the pancreas appeared atrophic (Figs. 6 and 7). Islets were very few and many islets were 80 to 90 per cent composed of alpha cells. These alpha cells, however, showed no remarkable changes in findings. The cytoplasm of the other remaining cells appeared transparent and contained many vacuoles.

In each instance, no change was recognized in the acinar cells of the pancreas and in the duct epithelium or in the walls of the blood vessels. No infiltration of polymorphonuclear leucocytes or other wandering cells was observed in necrotic tissues. Exclusively in Rabbit 8, in part of the pancreas circumscribed bleeding and cellular infiltration with many small round cells were seen, but these might be thought to be accidental changes.

Histologic Changes of the Pancreas in Dithizone Diabetes.—Islets of all the rabbits showed marked lesions. In the rabbit sacrificed two hours after intravenous injection of dithizone, the nuclei of the beta cells were stained faintly or deeply, and their cytoplasm appeared homogenous, with disappearance of granules, boundary of cells not being clear. Partially damaged islets were observed, but alpha cells remained almost normal in number and character.

Rabbit 59 which showed hypoglycemia after four hours and died after eight hours showed the most remarkable necrosis and disintegration of beta cells of the islets. The alpha cells situated at the periphery of the islets remained normal in number and character. Other tissues of the islets became as a whole homogenous, necrotic masses, stained faintly violet, in which a few shadows of nuclei or pyknotic nuclei and spaces around the nuclei were seen scattered. Most of the islets showed these lesions, but in a few relatively slightly damaged islets, there remained a few cells showing pyknosis of nuclei, disappearance of granules, and formation of vacuoles in cytoplasm. None of the islets was spared from the damage. All the islets showed more or less necrosis and disintegration. No infiltration of polymorphonuclear leucocytes or other wandering cells was observed in necrotic tissues.

Rabbits 62, 63, 64, and 67, which died ten to twenty hours after injection from hypoglycemia, showed almost similar changes (Figs. 8 and 9). While at the periphery of the islets alpha cells remained normal, the centers of the islets became a necrotic mass, stained faintly violet, with a few shadows of nuclei and pyknotic nuclei and spaces around the necrotic areas retained. Rabbit 64 showed slight lesions and a few cells which relatively preserved their structure and in which nuclei remained pyknotic.

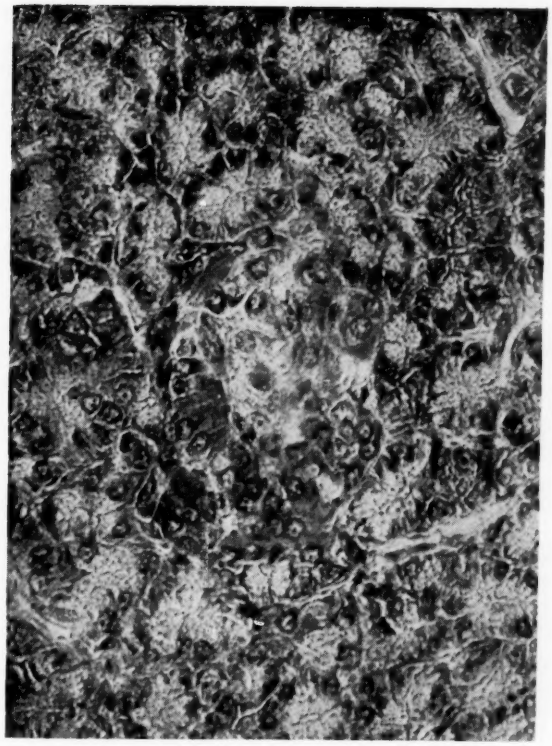


Fig. 6.—Islet of Langerhans of Rabbit 18 developing continuous permanent diabetes during 120 days after intravenous injection of oxine. Islet is reduced in size and composed chiefly of alpha cells and fibrous structure. (Gomori's chromium hematoxylin phloxin stain.)

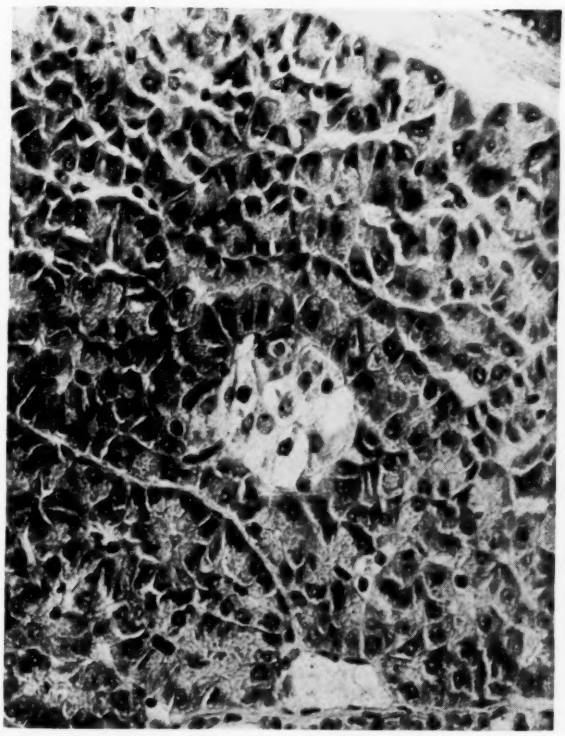


Fig. 7.—Islet of Langerhans of Rabbit 18 continuing permanent diabetes for 120 days by injection of oxine. Islet is markedly reduced in size and remaining cells contain many hydropic vacuoles.

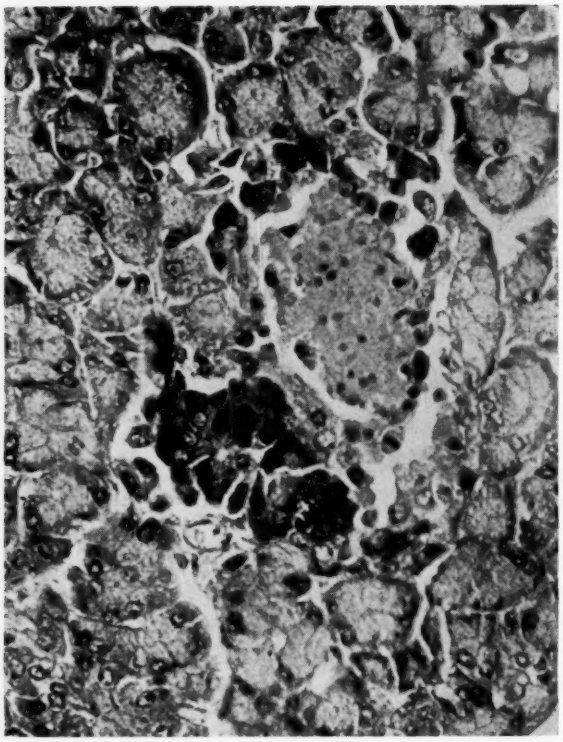


Fig. 8.—Islet of Langerhans of Rabbit 67 which died from hypoglycemia ten hours after intravenous injection of dithizone. Center of islet shows severe necrosis forming homogenous mass in which still shadows of nuclei are observed. But alpha cells at periphery of islet appear normal. (Gomori's chromium hematoxylin phloxin stain.)

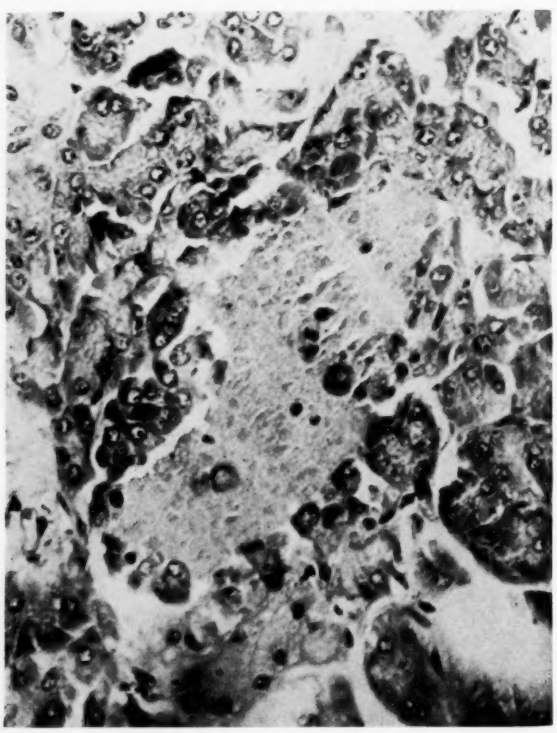


Fig. 9.—Islet of Langerhans of Rabbit 62 which died from hypoglycemia fifteen to twenty hours after injection of dithizone. While alpha cells remain normal at periphery of islet, the center has become a homogenous necrotic mass in which only a few pyknotic nuclei remain.

In Rabbit 58, sacrificed after twenty-four hours, alpha cells appeared normal but the cytoplasm of the beta cells appeared cloudy or netlike, stained pale red-violet. Their boundary was not distinct; they contained many vacuules and their nuclei were pyknotic or necrotic, stained reddish.

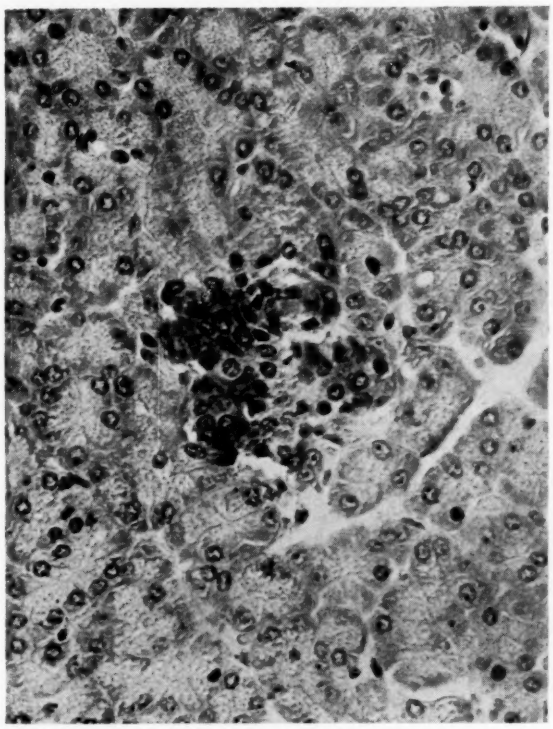


Fig. 10.—Islet of Langerhans of Rabbit 66 sacrificed five days after intravenous injection of dithizone. Islet is reduced in size and composed chiefly of alpha cells with a few pyknotic nuclei. (Gomori's chromium hematoxylin phloxin stain.)

In Rabbit 48 after forty-eight hours, alpha cells increased and were located at the periphery of the islets. Some islets were composed chiefly of alpha cells. The beta cells appeared transparent and of a cloudy or netlike structure with absence of granules, containing faintly stained nuclei and fine vacuoles.

In two rabbits having permanent diabetes, killed after five (Rabbit 66) and eight days, the islets were reduced in number and size (Fig. 10). These islets, mainly composed of alpha cells, appeared normal except a few pyknotic nuclei and a fibrous structure. Rarely, a few agranulated cells were observed. These findings in permanent diabetes showed disappearance and atrophy of the islets, as the result of absorption of necrotic cells in the hypoglycemic phases.

In dithizone diabetes, the pancreas apart from the islets showed no change. No change in blood vessels or duct epithelium and no infiltration of polymorphonuclear leucocytes or other wandering cells in the necrotic areas were observed.

Histologic Changes in Other Tissues.—Following the intravenous injection of oxine, the hearts of all the rabbits showed congestion of the small vessels, capillary bleeding, and a widespread or small infiltration of cells in the interstitium of the myocardium. These infiltrating cells were chiefly composed of myocytes having owl-eyed nuclei and basophilic cytoplasm and mixed with a few small round cells. In these areas, necrosis and bleeding were also observed.

In the early stages of oxine and dithizone diabetes, deposition of large amounts of glycogen was observed in all acini of the liver. But no glycogen was found in permanent diabetes of long duration. It was observed that the Kupffer cells and then Glisson's capsules contained large amounts of fat in oxine diabetes. In dithizone diabetes the Glisson capsules showed the same finding.

In the kidney no remarkable lesions were observed in either type of diabetes. But the tubular epithelium in permanent oxine diabetes (120 days) appeared quite transparent. The cells contained only nuclei and a large amount of glycogen. In this case the blood in the glomeruli and blood vessels was stained orange-red by sudan III staining, corresponding to the findings of lipemia. In other tissues and endocrine organs remarkable changes were not observed.

Histochemical Changes of Zinc in the Islets of Langerhans and in Other Tissues.—Histochemical findings of zinc in islets at varying intervals after injection of oxine and dithizone were as follows. Six hours after injection of oxine, the zinc reaction in the islets appeared almost normal and the whole islet cells were stained reddish-violet; some were deeply stained. In the specimens of rabbit which showed no hypoglycemia after twenty-four hours, zinc was slightly decreased and was located at the periphery of the islets. In the rabbit which died after eighteen to thirty-five hours from hypoglycemic convulsion, necrotic masses in the centers of the islets showed slight zinc reaction, that is, faintly reddish stain, whereas the peripheral area was stained only pale reddish. In forty-eight to seventy-two hours the whole islet cells showed only a slight zinc reaction. Ten days after the production of diabetes, the zinc reaction was negative. In permanent oxine diabetes continuing for 120 days, the reduced islets showed a slight zinc reaction.

Summarizing these findings, we could ascertain that zinc in the islets of Langerhans decreased in the course of time, was slightly positive in two to three days, and finally became negative. It was not clear which cells, alpha cells or beta cells, showed zinc reaction, but it was observed that the whole islet stained purplish-red, and one part of the islets showed intense zinc reaction. In islets in the hypoglycemic phases, cells were not distinct, and the necrotic substances showed a slight zinc reaction. In cases where zinc reaction was nearly normal, zinc was demonstrated as reddish-violet granules, but in other instances zinc was shown as having a homogenous purple-red color. Two hours after injection of dithizone, zinc in the islets was reduced slightly and was demonstrated mostly at the periphery of the islets. In Rabbits 59 and 66, in the hypoglycemic phase, zinc reactions were observed slightly reddish, with pale-stained nuclei amid necrotic masses in the centers of islets. Rabbits 62,

64, and 67, in the hypoglycemic phase, showed no zinc reaction in the islets with a few slightly positive islets. In general, zinc reactions were markedly reduced or almost negative in the hypoglycemic phase. In Rabbit 58, twenty-four hours after injection, in the permanent hyperglycemic phase, zinc reactions were more reduced and necrotic cells in the centers of the islets showed a slight zinc reaction. Rabbit 48, sacrificed at forty-eight hours, showed small and a few necrotic islets but no zinc was demonstrated. In Rabbits 45 and 66, killed after three and five days, islets were decreased and reduced in size, and zinc reactions were negative. In Rabbit 60, after eight days, most of the islets presented no zinc reaction, but in a few islets zinc was observed slightly positive, especially in a few cells with pyknotic nuclei. As the zinc reactions were reduced in all the rabbits, usually zinc was found not as granules but as faintly reddish stain.

In the intestines, submaxillary gland, and prostate in which zinc is detected in normal animals, histochemical changes of zinc were studied in oxine and dithizone diabetes. The zinc reaction in the submaxillary gland remained normal or was increased. Relations between zinc reactions and lapse of time after injection of reagents were not recognized. Only in permanent oxine diabetes a large amount of zinc was found in granular form. In other rabbits it appeared diffusely stained and no difference was observed between acinar cells and duct epithelium. In the intestines zinc reactions were reduced in general, but there was no close relation between zinc reactions and intervals after injection. But most of the animals sacrificed early showed negative zinc reactions. Only Rabbit 40 forty-eight hours after oxine injection showed a marked deposition of zinc in the intestines. The prostate showed a slight positive or negative reaction of zinc, with no relation to the intervals after injection. In other tissues zinc was not observed histochemically.

DISCUSSION

Based on the observation by Okamoto that zinc can be detected histochemically in the islets of Langerhans, it may be suggested that zinc reagents may cause the destruction of islet cells and diabetes may be produced. With this idea in mind, six organic reagents—oxine (8-hydroxyquinoline), dithizone (diphenylthiocarbazone), quinaldinic acid, anthranilic acid, 4-hydroxybenzthiazol, and diphenylthiocarbazide—were injected intravenously into rabbits. Among them, oxine and dithizone could cause typical blood sugar changes in the three phases: initial hyperglycemia immediately after injection, followed by hypoglycemia and secondary hyperglycemia, and finally by permanent diabetes. In the case of oxine, initial hyperglycemia occurred in one to four hours after injection, hypoglycemia in ten to twenty-four hours, and permanent hyperglycemia in forty-eight hours. In the case of dithizone, initial hyperglycemia occurred in one to two hours after injection, hypoglycemia in eight to twelve hours, and permanent hyperglycemia in twenty-four hours. In severe hypoglycemia in both diabetes, hypoglycemic convulsions occurred and

continued for a few hours, followed by death when the rabbit was untreated. But most of the rabbits recovered when administered a total dose of 20 to 30 Gm. glucose.

Other reagents caused no fluctuation of blood sugar except a transitory hyperglycemia by diphenylthiocarbazide. Differences in the influence of these reagents upon the blood sugar and in their diabetogenic actions may be due to differences in their avidity for zinc.

Oxine and dithizone, which are very sensitive in their reaction with zinc in a wide range of pH of reacting solvents, showed an intense diabetogenic action. On the contrary, it seems that quinaldine acid, anthranilic acid, 4-hydroxybenzthiazol, and diphenylthiocarbazide possess no diabetogenic action, as they have weak damaging effect on tissues and are not sensitive in reaction with zinc in solutions which can be injected into the body.

Comparing the diabetogenic action of oxine and dithizone with that of alloxan, initial hyperglycemia in dithizone diabetes is remarkable and hypoglycemia occurs earlier than in oxine diabetes; changes of blood sugars are similar to those produced by alloxan. It may be accepted that in dithizone diabetes the degree of permanent hyperglycemia and diabetes is more intense than in oxine diabetes and corresponds to that of alloxan. Besides, it was proved that dithizone caused hyperglycemia and diabetes by oral administration. This may suggest that dithizone can be absorbed in the intestines as it is soluble in alkaline solution and reaches the islets by the blood stream. Hitherto, there has been no research done on the changes of blood sugar induced by these organic reagents. As for anthranilic acid, Mitsuba and Ichihara[29] reported that after administration of anthranilic acid in rabbits and dogs, reducing substances appeared in the urine; but they presumed that these might be connected with glucuronic acid since they are negative in fermentation reactions.

In our experiments, it was confirmed that oxine and dithizone could cause the typical three phases of changes in blood sugar and permanent diabetes, and reducing substances in the urine were proved to be glucose by means of fermentation and osazone reactions. The diabetogenic action of these reagents may be due to their linking to zinc in the islets and the destruction of the islet cells, since they are different types of compounds and have no common properties except their reaction with zinc. From the fact that the three phases of blood sugar changes by oxine and dithizone are very similar to those caused by alloxan, it may be suggested that these changes may be caused by a similar mechanism. This is also confirmed by the histologic changes in the islets of Langerhans.

Already early after the injection of oxine, lesions in the islets were observed. In six to twenty-four hours in the initial hyperglycemic phase, slight degenerative changes and necrosis of beta cells were found. Nuclei were pyknotic or stained faintly, and the cytoplasm showed disappearance of ganules. Some islets became homogenous, and the boundary of the cells was not clear. But alpha cells remained almost normal in number and appearance of nuclei and cytoplasm. In the hypoglycemic phase, a most conspicuous necro-

sis and disintegration of beta cells was observed. While alpha cells at the periphery of the islets were preserved and appeared normal, the centers of the islets became a homogenous, necrotic mass which was stained faintly and showed a complete destruction of structure with a few necrotic nuclei or shadows of nuclei. In slightly damaged islets, degenerated or necrotic cells were seen scattered in the islets. In the secondary hyperglycemic phase and in permanent diabetes, damaged beta cells disappeared and the islets were decreased and mostly composed of alpha cells. In persistent diabetes for a long period, the islets were markedly reduced and decreased and composed almost of alpha cells and a few cells with many vacuoles. In the initial hyperglycemic phase at two hours after injection of dithizone, beta cells of the islets showed pathologic changes such as pyknosis of nuclei, disappearance of granules, and homogenization of cytoplasm and necrosis, whereas the alpha cells remain normal. In eight to twenty hours in the hypoglycemic phase, the changes of the islets were most conspicuous. While alpha cells were almost normal and located at the periphery of the islets, beta cells in the center of the islets showed striking degenerative changes. Necrosis and disintegration and homogenous necrotic masses were observed with necrotic or pyknotic nuclei. In secondary hyperglycemia and permanent diabetes, islets were decreased and reduced. Most of the islets were composed of alpha cells, and only a few degenerated beta cells remained. In both diabetes, inflammatory reactions, such as infiltration of leucocytes or other wandering cells, were not observed. Woerner[42] reported that continuous injection of glucose in a guinea pig caused exhaustion of granules in beta cells after twenty-four to forty-eight hours, and then increase of the islets and hydropic degeneration and degranulation of beta cells after two to four weeks.

In the islets of Langerhans of cats and dogs with diabetes produced by partial pancreatectomy, degeneration and disappearance of beta cells were reported by Homans[19] and others. In pituitary diabetes, Richardson[35, 36] and others reported degranulation, degeneration of beta cells, and decrease and atrophy of the islets in permanent diabetes. In alloxan diabetes, quite selective degeneration and necrosis were confirmed by many investigators[3, 10] since the first discovery by Dunn and others.[11]

From these facts, it may be accepted that beta cells in the islets have a close relation with carbohydrate metabolism and are the cells which produce insulin. As mentioned, pathologic changes in the islets in oxine and dithizone diabetes are quite similar to those in alloxan diabetes. It may be concluded that oxine and dithizone as they are strong zinc reagents, when injected into the blood stream reach the pancreas and connect with the zinc in the islet cells and destroy the cells, and that permanent diabetes could be produced as the result of destruction of the islets.

This presumption may also be confirmed by histochemical findings of zinc in the islets. If oxine and dithizone injected into the blood stream connect and react with zinc in the islets, it may be expected that histochemical zinc reactions in the islets are reduced after injection of oxine and dithizone. In fact this was confirmed, as already described. Zinc reactions declined in

course of time after injection of oxine and dithizone. In initial hyperglycemia, zinc reactions were slightly reduced, but they were almost absent in the hypoglycemic phase and completely absent in the diabetic state. Accordingly, these histochemical findings of absence of zinc in the islets in oxine and dithizone diabetes might be interpreted as having a close relation to the content or secreting dose of insulin in the islets. In the submaxillary gland, intestines, and prostate in which zinc can be found in normal animals, zinc reactions were generally reduced after injection of oxine and dithizone. Only the submaxillary gland in permanent oxine diabetes contained a large amount of zinc. The physiologic action of zinc in these tissues is not clear, and in our experiment it was only recognized that zinc reactions were reduced without any remarkable lesion.

The mechanism by which the characteristic changes of blood sugar are produced by oxine and dithizone is discussed below as compared with that by alloxan. Initial hyperglycemia by alloxan has been interpreted as the result of irritation of the adrenal gland, the release of adrenalin (Goldner and Gomori,[15] and Hughes, Ware, and Young[22]) or the direct action of alloxan on the liver and the stimulation of gluconeogenesis (Houssay, Orias, and Sara[21]). From the fact that oxine and dithizone are very sensitive in their reaction with zinc and that they reach the islets in a few minutes after injection, it may be concluded that initial hyperglycemia may be caused by the temporary cessation of insulin secretion as the result of the connection of the reagents with zinc in the islets.

It is supposed that hypoglycemia by alloxan may be caused by temporary release of large amounts of insulin (Goldner and Gomori[15]) or by lack of glucose production in the liver (Houssay, Orias, and Sara[21]). In hypoglycemia by oxine and dithizone, the most remarkable necrosis and disintegration of the islets are observed, and the zinc reaction is slightly positive in these necrotic tissues. For recovery from severe hypoglycemia it is necessary to administer 20 to 30 Gm. glucose, which corresponds to the whole amount of insulin in a rabbit. Further hypoglycemia is remarkable in animals in which zinc is detected in a large amount but not conspicuous in animals in which only a small amount of zinc is detected. From these facts it may be suggested that hypoglycemia by oxine and dithizone may be caused by the liberation of insulin from the destructed islets. Final hyperglycemia by alloxan is attributed by many investigators[10, 15, 24] to the decrease or cessation of secretion of insulin, as the result of the destruction of beta cells. Final hyperglycemia in oxine and dithizone diabetes may also be assumed to be the result of lack of insulin, as may be concluded from the histologic changes and the negative zinc reaction in the islets.

SUMMARY

1. With the expectation that organic reagents, which react with zinc in the islets of Langerhans, may cause the destruction of the islet cells and produce diabetes, the six reagents were injected into rabbits.

2. By intravenous injection of 50 mg. per kilogram oxine, the three phases of blood sugar changes were observed. Initial hyperglycemia in one to four hours, followed by hypoglycemia in ten to twenty-four hours, and finally permanent diabetes after forty-eight hours were observed.

3. By injection of the same doses of oxine few rabbits showed transitory hyperglycemia which returned to normal value after twenty-four to forty-eight hours. Intravenous injection of 10 to 20 mg. per kilogram oxine caused only transitory hyperglycemia.

4. By intravenous injection of 50 to 200 mg. per kilogram dithizone in 0.2 to 0.5 per cent ammoniac solution, the three phases of blood sugar changes were observed, namely, initial hyperglycemia which reached its peak after one to two hours, followed by hypoglycemia in eight to twelve hours, and then gradual rise of blood sugar and permanent diabetes after twenty-four hours. Oral administration of 500 mg. per kilogram dithizone caused transitory hyperglycemia which continued for two to three days.

5. Quinaldinic acid, anthranilic acid, and 4-hydroxybenzthiazol caused no changes of blood sugar. After injection of diphenylthiocarbazide, a tendency to slight elevation of blood sugar was observed.

6. Histologic changes of the pancreas were observed by Gomori's staining method at various intervals after intravenous injection of oxine and dithizone. In the initial hyperglycemic phase in six to twenty-four hours after injection of oxine, slight degenerative changes and necrosis were observed in the islets of Langerhans. The beta cells showed pyknotic or faintly stained nuclei and disappearance of granules in the cytoplasm, but alpha cells remained almost normal. In the hypoglycemic phase in eighteen to thirty-five hours, marked necrosis and disintegration of beta cells were observed. Centers of the islets became necrotic masses, but alpha cells at the periphery of the islets remained normal. In the final hyperglycemia after forty-eight hours and in permanent diabetes, degenerated beta cells were reduced and the islets were composed of alpha cells. In permanent diabetes of long duration, the islets were reduced in size and number and consisted of alpha cells.

7. In initial hyperglycemia two hours after intravenous injection of dithizone, islets showed necrosis of the nuclei and disappearance of granules and necrosis of beta cells. In the hypoglycemic phase in eight to twenty hours, beta cells in the centers of the islets showed marked necrosis and disintegration, forming necrotic homogenous masses with shadows of nuclei, whereas surrounding alpha cells remained almost normal. In the final hyperglycemia after twenty-four hours and in permanent diabetes, the islets were reduced, appearing small, and were composed chiefly of alpha cells with a few degenerated cells. In both kinds of diabetes, inflammatory reactions were not observed in the necrotic tissues of the islets.

8. Remarkable changes were not observed in other tissues in oxine and dithizone diabetes.

9. In the initial hyperglycemic phase of oxine and dithizone diabetes, histochemical zinc reactions in the islets were slightly reduced and were further reduced in the hypoglycemic phase. In the final hyperglycemic

phase zinc was not found in the islets but was found slightly positive in permanent diabetes of long duration. These changes of zinc in the islets may be considered as changes of insulin content in the islets.

In the submaxillary gland, intestines, and prostate in which zinc is found in normal rabbits, zinc reactions generally were reduced. Much zinc was found in the submaxillary gland in permanent oxine diabetes of long duration.

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THE EFFECT OF POTASSIUM DEFICIENCY ON CARBOHYDRATE METABOLISM

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THE therapy of electrolyte disturbances is becoming increasingly oriented toward correction of fundamental imbalances in the composition of intracellular fluid.[1-4] Evidence pointing toward deficiency of intracellular potassium has been found in patients who have become depleted as a result of diarrhea,[5-7] loss of fluid from the gastrointestinal tract by vomiting or drainage,[8-13] ulcerative colitis,[14] diabetes mellitus,[15-17] and overproduction of the desoxycorticosterone type of hormone in cases of adrenal cortical hyperplasia or cancer.[18] A considerable number of in vitro studies indicate the important intracellular role of potassium. Evidence suggests that phosphocreatine exists in skeletal muscle as a dipotassium salt.[19] Phosphorylation of the adenylic acid system,[20, 21] the activity of choline acetylase,[22] and optimum glycogenesis in liver slices[23] require the presence of potassium. For rational treatment of clinical intracellular depletion, further information is desirable concerning the interaction of intra- and extracellular electrolytes with the organic compounds of cellular metabolism.

The experiment to be reported was designed to test in the intact animal the effect of intracellular potassium deficiency on the metabolism of glycogen and glucose. The technique of biologic equilibrium, as devised by Darrow,[1] was used. This involves the deprivation of dietary potassium for a period of weeks, thus permitting the development of any compensatory phenomena.

Rats were therefore subjected to dietary potassium deprivation for lengths of time ranging up to 120 days. Their state of carbohydrate metabolism was assayed by determinations of urinary glucose, oral glucose tolerance, and liver and muscle glycogen content. Tissue electrolytes were determined to establish quantitatively the extent of potassium deficiency. The results were compared with similar determinations on rats receiving the same basal diet plus adequate potassium.

The results obtained indicate that potassium deficiency in the intact animal has dual effects on carbohydrate metabolism. One effect becomes evident relatively early and apparently involves overproduction of adrenal cortical hormones. When potassium lack is very advanced, there is, in addition, absence of tissue glycogenesis.

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MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain were used, weighing 160 to 175 grams at the beginning of the experiment. A low potassium synthetic diet* was used to which potassium was added for the control rats. Composition of the diet with respect to sodium, potassium, and chloride is shown in Table I. The rats were divided into three groups: A, rats fed the synthetic diet plus potassium; B, rats kept on the potassium-deficient diet 40 to 60 days; and C, rats kept on the potassium-deficient diet 90 to 120 days.

TABLE I. COMPOSITION OF DIETS PER 100 GM.

DIET	Na (mM.)	K (mM.)	Cl (mM.)
Low K	30.4	0.07	8.0
Added K	30.4	3.80	8.0

During the period on the deficient diet, numerous oral glucose tolerance tests were done. The rats were fasted overnight, and next morning were given 10 Gm. per kilogram glucose (as 50 per cent glucose in water) by stomach tube. Blood samples were taken from the tail before the glucose was given, and two, four, and six hours afterward. Total eosinophil counts without anesthesia were done on blood from the tail by the method described by Uribe and co-workers.[24] At the time of sacrifice, rats were fasted and fed glucose as for the glucose tolerance tests, and at the four-hour point the rats were anesthetized with 25 mg. per kilogram Amytal intraperitoneally, decapitated with scissors, and bled into centrifuge tubes. Amytal was used to avoid convulsions caused by decapitation, since these might have affected muscle glycogen. Amytal has been shown to have little effect on tissue glycogen.[25] The interval of four hours was chosen because Cori and associates[26] found that rat liver glycogen is at a maximum four hours after an excess of glucose is given orally.

Whole blood for sugar determination was collected in a tube containing fluoride. The remaining blood was collected in another tube and centrifuged to separate the serum. Samples of thigh muscle and of liver for glycogen determination were transferred to 35 per cent potassium hydroxide solution within sixty seconds and immediately digested. The remaining thigh muscle was stripped of fascia and nerves and introduced into tared tubes for electrolyte analysis. The liver was blotted free of blood and introduced into tared tubes. A 0.5 Gm. sample of liver was placed in 0.1 per cent sodium carbonate solution for total blood estimation. Both adrenals were placed in a small tared tube and weighed as a pair on a chainomatic balance.

The following chemical methods were employed. (a) Tissue glycogen, a modification of the method of Good, Kramer, and Somogyi.[27] Glucose was determined after acid hydrolysis by the macromethod of Folin[28] and glycogen was expressed as glucose, grams per 100 grams wet tissue; (b) blood glucose method of Folin[29]; (c) serum chloride method of Wilson and Ball[30]; (d) tissue chloride, method of Wallace and Hastings[31]; (e) serum water, gradient tube method of Linderstrøm-Lang, as modified by Lowry and Hunter.[32] Tissue was prepared for estimation of cations by drying to constant weight in a 110° oven; it was ground to a powder in a mortar and ashed with 10N sulfuric acid. After evaporation on a steam bath the sample was heated overnight in a Muffle oven at 500° C. and transferred to a volumetric flask with warm 0.1N hydrochloric acid. Cations were determined on aliquots of the latter as follows: (a) potassium, method of Fiske and Litarczek[33]; sodium, method of Butler

*The low potassium synthetic diet was made up as follows (grams per 100 Gm. diet): (1) Devitaminized casein (Sheffield Farms), 25; (2) corn oil, 4 (extracted with warm 2 per cent hydrochloric acid); (3) U.S.P. dextrose, 66; (4) salt mixture: $CaCO_3$, 1.34; $CaHPO_4.2H_2O$, 0.238 NaCl, 0.468; Na_2HPO_4, 1.59; $MgSO_4$. $7H_2O$, 1.016; NaI, 0.0003; $CuSO_45H_2O$, 0.004; $ZnCl_2$, 0.0008; $MnSO_4.4H_2O$, 0.0324; iron and ammonium citrate, 0.296; K_2HPO_4, 0.335 was added to this diet for feeding the control rats. Growth factors were added as follows (per 100 Gm. diet): thiamine chloride, 400 μg; riboflavin, 800 μg; pyridoxine hydrochloride, 400 μg; calcium pantothenate, 2.5 mg.; choline chloride, 100 mg.; vitamin A, 25 I.U.; vitamin D, 25 I.U.; vitamin E, 1 mg.; cystine –L–(–), 200 mg.; nicotinic acid, 10 mg.; methyl naphthaquinone, 10 milligrams.

TABLE II

SUMMARY OF MEASUREMENTS ON SKELETAL MUSCLE, LIVER, SERUM, AND BLOOD ON CONTROL (GROUP A) RATS AND ON

GROUP	NUMBER OF RATS	DAYS ON DIET	DIET*	SKELETAL MUSCLE							
				CONC. PER 100 GM. FAT FREE SOLIDS						CONC. PER 100 GM. WET TISSUE	
				Cl (MEQ.)	Na (MEQ.)	K (MEQ.)	Ca (MEQ.)	Mg (MEQ.)	TOTAL BASE (MEQ.)	H_2O (GM.)	GLYCOGEN (GM.)
A	11		N	3.56	8.7	36.5	2.0	6.4	53.6	75.5	0.36
(S.D.)				0.8	1.4	0.9				1.2	.22
B	8	40-60	LK	4.15	18.2	24.9	3.4	7.8	54.3	75.4	1.53
(S.D.)				0.8	2.9	2.1				2.1	.93
C	6	90-120	LK	3.97	20.4	23.8				76.3	0-0.23
(S.D.)				1.2	1.5	3.1				1.1	

*N, normal diet; LK, low potassium diet.

†Counts were made on six Group A, eight Group B, four Group C rats. The rats had free access to food when the counts were made.

and Tuthill[34]; calcium, method of Logan[35]; magnesium, method of Briggs.[36] Total blood in liver was estimated by a modification of the method of Lowry and Hastings.[37] Tissue neutral fat was determined by the method of Hastings and Eichelberger.[38]

RESULTS

Rats fed the potassium-deficient diet gained weight slowly for 35 to 40 days, after which time gradual weight loss, anorexia, and diarrhea were manifested. Several of the rats in the 90 to 120 day group died suddenly and so were not included in the experiment. The control rats that received the deficient diet plus added potassium grew better than a control group fed Purina chow.

Table II compares the electrolyte composition of skeletal muscle and liver in control and potassium-deficient animals together with data on liver and muscle glycogen, serum chloride, blood glucose, and total circulating eosinophils.

(a) Skeletal Muscle Electrolyte Changes.—The data of Table II show that the concentration of calcium and magnesium in the muscle of potassium-deficient Group B is increased over the control values. Although this is contrary to the findings of Orent-Keiles and McCollum,[39] this result is not surprising because of the much lower concentration of dietary potassium available to our rats. Previous experiments by Fenn and Haege using excised frog muscle[40] and Buell and Turner using intact rats[41] have indicated that magnesium and potassium compete for place in the intracellular fluid of muscle. The increase of calcium and magnesium in the skeletal muscle of potassium-deficient rats has also been confirmed by analyses on individual animals in another experiment.[42]

The data of Table II uphold the findings of other investigators regarding the increase of muscle sodium and decrease of muscle potassium in dietary potassium deficiency.[1, 39, 43]

When Groups B (potassium deficient 40 to 60 days) and C (potassium deficient 90 to 120 days) are compared with respect to muscle electrolyte composition, no marked differences are noted between the mean values for

II
AND ON RATS FED A POTASSIUM-DEFICIENT DIET FOR 40 TO 60 DAYS (GROUP B) AND 90 TO 120 DAYS (GROUP C)

LIVER								SERUM				
CONC. PER 100 GM. FAT FREE SOLIDS						CONC. PER 100 GM. WET TISSUE		CONC. PER LITER		BLOOD GLUCOSE (MG. 100 ML.)		EOSINOPHILS†
Cl (MEQ.)	Na (MEQ.)	K (MEQ.)	Ca (MEQ.)	Mg (MEQ.)	TOTAL BASE (MEQ.)	H_2O (GM.)	GLYCOGEN (GM.)	H_2O (GM.)	Cl (MEQ.)	FASTING	4 HR. POSTPRANDIAL	NUMBER PER CU. MM.
7.2	7.1	32.4	2.0	4.8	46.3	69.3	2.47	932	100.0	104.0	122.0	347
1.4	0.4	1.4				0.3	0.3	6	0.8	15.0	21.0	
8.1	12.2	33.3	2.8	7.0	55.3	72.7	6.56	943	83.6	99.0	102.5	115
1.7	1.2	3.8				1.9	0.9	3	5.2	20.0	23.2	
6.8	11.9	29.6				72.8	0-0.53	931	81.3	110.0	555.3	0-9
0.9	1.1	3.3				2.3		11	5.5	18.0	221.0	

chloride, sodium, and potassium, although the lowest values for muscle potassium were found in two rats of Group C.

(b) Liver Electrolyte Changes.—Analysis of the livers for potassium bears out previous observations[43] that in general severe potassium deficiency does not cause diminution of liver potassium. However, there were two rats in both Group B and Group C which had liver potassium values between 25.3 and 28.7 meq. per 100 grams of fat-free solids, compared with the mean control values of 32.4 ± 1.4 meq. It is noteworthy that liver sodium values were significantly higher in the potassium-deficient group.

These data also demonstrate the disproportionately high ratio of chloride to sodium in the normal liver (Group A), in confirmation of previous observations.[43] The calcium and magnesium values for potassium-deficient liver are increased over the controls, but since these determinations were done on pooled liver from several rats, it is not possible to evaluate their validity on a statistical basis.

(c) Other Changes.—Although all the rats received a diet adequate in chloride, the values for serum chloride in the potassium-deficient rats are uniformly low, as others have shown previously.[1, 43]

Liver and muscle glycogen values in the rats of Group B are more than double control values, whereas the rats of Group C show only traces of glycogen either in liver or muscle. Groups A and B show similar values for blood glucose, and repeated oral glucose tolerance tests on these rats revealed normal curves. In Group C terminal blood glucose values four hours after glucose was given by mouth are strikingly elevated with a mean value of 555 ± 221 mg. per cent and a maximum value of 900 mg. per cent. None of the rats of Groups A, B, or C had *fasting* blood glucose values of more than 140 mg. per cent, and no glucosuria was observed in the fasting state.

Fig. 1 shows an oral glucose tolerance test on a rat of Group C compared with a test on a control rat and with the blood glucose curve of diabetes due to pancreatectomy and that of steroid diabetes (two latter curves from data of Ingle.[44]

Table II shows blood total eosinophil counts done on control and potassium-deficient animals. There is a marked fall in circulating eosinophils in severe potassium deficiency.

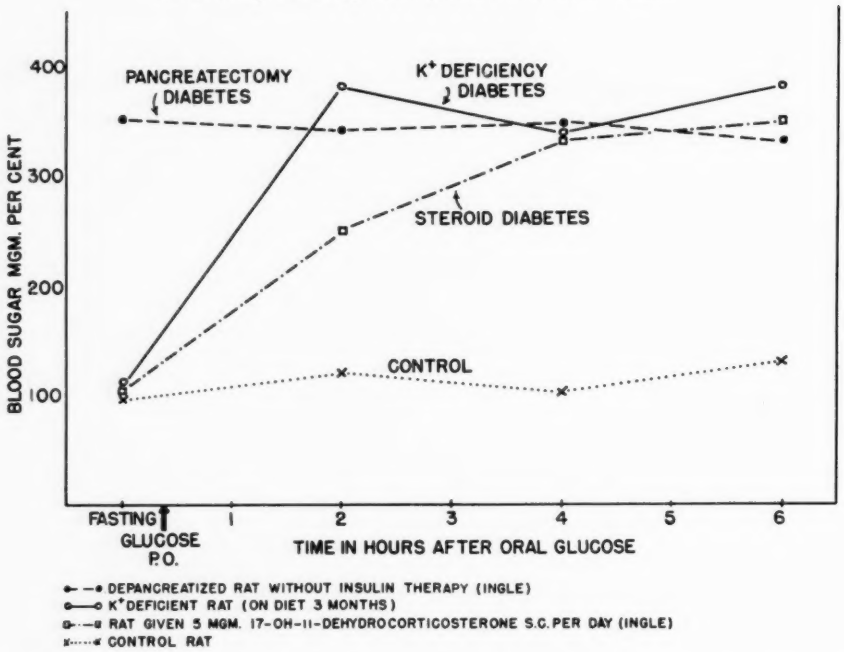

Fig. 1.—Oral glucose tolerance curves in a control and a potassium-deficient (Group C) rat compared with glucose tolerance curves done by Ingle[11] on a depancreatized rat and on a rat injected with 17-OH-11-dehydrocorticosterone.

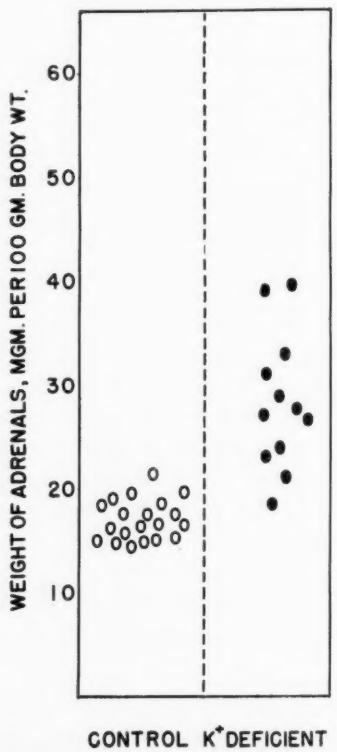

Fig. 2.—Comparison of adrenal weights in control rats (Group A) and potassium-deficient rats (Groups B and C). The values given represent the weight of both adrenals expressed per 100 grams body weight.

Adrenal weights expressed as milligrams per 100 grams of body weight are shown in Fig. 2. Weights for glands from both Groups B and C are given together, since there was no apparent difference between their weights. The data indicate an increase in the weights for both groups of the potassium-deficient animals, a finding also described by Deane, Shaw, and Greep.[45] Fig. 3 shows the temporal relationships of the principal data relating to carbohydrate abnormality in potassium deficiency. The decrease of circulating eosinophils and tissue glycogen on one hand and elevation of blood glucose on the other should be noted.

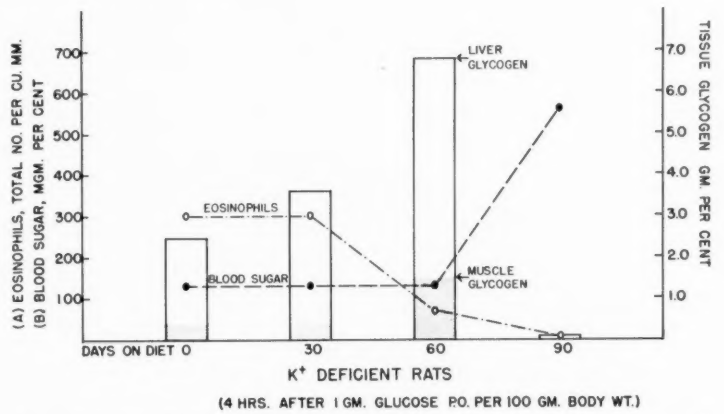

Fig. 3.—Composite diagram illustrating relationships between concentrations of liver and muscle glycogen, blood sugar, and total circulating eosinophils in control and potassium-deficient rats.

Rats Potassium Deficient for 40 to 60 Days (Group B).—These animals showed abnormally high concentration of liver and muscle glycogen. It is possible that moderately severe potassium deficiency interferes with some glycogenolytic enzyme system. Our studies throw no light on this possibility. Another explanation for the increased tissue glycogen levels in Group B is increased glycogenesis. The situation obtaining in the animals of this experiment, in which both liver and muscle glycogen were elevated and in which there was evidence of adrenal cortical hyperactivity, is reminiscent of the experiments of Britton and Silvette.[46] These authors gave adrenal cortical extract to a large series of normal and adrenalectomized animals and found a rise in blood sugar and in liver and muscle glycogen. Further amplification of this problem by Long, Katzin, and Fry[47] revealed that there was a reduction of glucose oxidation in the tissues of normal, glucose-fed rats given adrenal cortical extract when compared with untreated controls. This was done by placing the rats in a Haldane respiration device and measuring the respiratory quotient. A relatively increased amount of the absorbed glucose was found to be stored as liver glycogen, and there was an absolute increase in the total carbohydrate content of the animal.

An interesting discrepancy exists between the work of these two groups, since the former workers found that adrenal cortical extract increased both liver and muscle glycogen in the normally fed animal, whereas the latter

group found an increase of muscle glycogen in mice but not in rats. The animals of Britton and Silvette were killed by a blow on the head, and those of Long, Katzin, and Fry were dissected under Nembutal anesthesia, which may account for the difference. Our rats, like those of Britton and Silvette, showed an increase of both liver and muscle glycogen and were killed by decapitation under Amytal anesthesia.

One explanation of our findings would be that the increase of liver and muscle glycogen in the Group B rats is due to increased secretion of adrenal glycogenic hormone. Supporting this idea is the finding that total circulating eosinophils in Group B were lower than in the controls (see Table II), which recent evidence suggests may be associated with increased production of adrenal cortical 11-17-oxycorticosteroids.[48]

Deane, Shaw, and Greep[45] have presented histochemical evidence suggesting that the production of adrenal desoxycorticosteroids is depressed in the potassium-deficient rat. This may, in a relative manner, intensify the effect of the 11-17-oxycorticosteroids being produced by the same gland, since the two hormones appear to be mutually antagonistic in at least one respect, their influence on sodium retention and excretion.[44, 49]

Rats Potassium Deficient for 90-120 Days (Group C).—Marked hyperglycemia in response to ingested glucose, with almost no tissue glycogenesis, occurred in Group C. In addition, total counts showed that eosinophils had almost disappeared from the circulation of these animals (see Table II). The data presented in Table II concerning tissue electrolytes of Groups B and C do not show much difference between the two. Calcium and magnesium determinations were not done in Group C tissues.

The increased adrenal weights and nearly absent eosinophils hint at increased circulating 11-17-oxycorticosteroids. The postprandial hyperglycemia and the almost total absence of tissue glycogen suggest that there is some defect in intracellular fluid which precludes glycogenesis.

DISCUSSION

Potassium is quantitatively the most prominent cation of intracellular water, and much evidence has previously accumulated linking it with carbohydrate metabolism.

Pulver and Verzár[50] showed that the addition of sugar to fasting yeast cells in a dilute potassium medium resulted in the uptake of both carbohydrate and potassium by the cell. That this is true for the human red cell has been demonstrated by Harris.[51]

Recent work by Dixon indicates that this system also obtains for brain cells. During anaerobic metabolism of brain tissue, there is no increase of potassium in the nutrient medium. If, however, there is an absence of glucose, or if glycolysis is inhibited by fluoride, potassium leaks out of the cells.[52]

Hastings and Buchanan[23] found that optimum glycogenesis in liver slices required an artificial medium containing concentrations of potassium, magnesium, and sodium comparable to those occurring in intracellular fluid.

Fenn[53] observed that the ratio of potassium to glycogen in liver was constant no matter what concentration of glycogen was produced in the liver of intact rats.

Our finding of very low tissue glycogen concentrations in animals suffering from prolonged tissue potassium deficiency (Group C) would appear to be best explained, in view of the foregoing observations, on the basis of alterations in the electrolyte composition of intracellular fluid.

CLINICAL IMPLICATIONS

In 1923 Briggs and Koechig[54] observed that serum potassium and inorganic phosphorus concentrations fell upon the administration of insulin. In the following year it was postulated by Harrop and Benedict that the phosphate-carbohydrate compound in the liver existed as a potassium salt.[55] More recent clinical observations indicate that in the absence of endogenous production of the hormone insulin, there is a rapid preferential loss of intracellular potassium.[15, 16] Dangerously low levels of serum potassium may be produced in diabetes mellitus treated with insulin alone, presumably because of the sudden transfer of extracellular potassium and glucose into the cell for glycogenesis.[17]

These findings lead us to therapeutic considerations. Our data indicate that in severe potassium deficiency there exists postprandial hyperglycemia and inhibition of intracellular glycogenesis. It is therefore a possibility that the hyperglycemia of some cases of diabetic coma with so-called "insulin resistance" may well be due as much to rapid intracellular potassium depletion with blocked glycogenesis as to lack of the hormone insulin.

From the foregoing data the therapy of diabetic coma with intravenous potassium solution would appear to be justified on theoretical grounds alone. Actually there is increasing clinical evidence that low serum or intracellular potassium concentrations are associated with cardiac abnormalities and deaths in diabetic coma and that treatment with parenteral potassium solution promotes rapid recovery.[17, 56-58]

SUMMARY

The effects of severe potassium deficiency on the metabolism of glucose and glycogen in the intact animal have been examined. A diet containing 0.07 mM. potassium per 100 grams and 30.4 mM. sodium per 100 grams caused severe potassium deficiency in 160 to 175 gram rats.

Muscle electrolyte analyses revealed diminished potassium and increased sodium, calcium, and magnesium concentrations in the potassium-deficient animals.

Liver electrolyte analyses revealed diminished potassium values in only two animals, but liver sodium was significantly increased in all potassium-deficient rats.

Rats kept on the potassium-deficient diet for 40 to 60 days showed abnormally high postprandial liver and muscle glycogen concentrations, normal fasting blood glucose and urine glucose concentrations, normal oral glucose

tolerance curves, moderately diminished total circulating eosinophils, and enlarged adrenal glands. This is interpreted as indicating chronic alarm with increased production of adrenocortical hormones.

Rats kept on the potassium-deficient diet 90 to 120 days showed almost absent liver and muscle glycogen concentrations, normal fasting blood glucose and urine glucose concentrations, markedly elevated oral glucose tolerance curves, few or no circulating eosinophils, and enlarged adrenal glands. This is interpreted as indicating continued chronic alarm, with blocked glycogenesis due to cumulative deficit of body potassium.

CONCLUSIONS

1. Rats deprived of dietary potassium over long periods of time develop a chronic alarm reaction, with abnormal carbohydrate metabolism.

2. These effects may be related to the lowered concentration of potassium and increased concentrations of sodium, calcium, and magnesium in intracellular fluids.

3. The inhibited tissue glycogenesis of severe potassium deficiency may potentiate the diabetic state, in which rapid losses of intracellular potassium are known to occur. This is additional data supporting the use of parenteral potassium solution in the treatment of diabetic acidosis.

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THE INEFFECTIVENESS OF ENTEROGASTRONE IN MANN-WILLIAMSON DOGS

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IT HAS been recognized for many years that the entrance of fat, and to a lesser extent sugar,[1] into the duodenum results in decreased gastric motility and secretion. This inhibition of gastric activity is considered to be mediated by enterogastrone, a chalone produced by the small bowel.

A concentrated extract of enterogastrone, prepared from the mucosa of the small intestine of hogs, has been used by several investigators in the treatment of jejunal ulcer in Mann-Williamson dogs. Ivy and associates[2-6] found parenteral enterogastrone to be of definite benefit in preventing ulcer formation during a period of one year's treatment, and also of protective value for a variable period after treatment was stopped. Sandweiss and associates[7, 8] considered parenteral enterogastrone of no benefit, but found oral enterogastrone of some benefit.

No clear-cut evidence of significant suppression of acid-peptic or motor activity of the stomach by this enterogastrone preparation, in the dosage commonly used, has been presented. It has been suggested, however, that its main effect is to increase the resistance of the jejunal mucosa to ulceration.[3, 4, 5]

METHODS

The material for presentation here is a summary of experiences with oral and parenteral enterogastrone therapy* in Mann-Williamson dogs at the University of Michigan.

Young, healthy, 10 to 20 kilogram dogs were selected and placed on a standard diet consisting of dog biscuits, canned dog food, evaporated milk, and dextrose. Canned fish and fresh meat supplemented this diet. The Mann-Williamson procedure was carried out in the usual manner.[9] Those animals scheduled to receive parenteral enterogastrone were started on such therapy immediately after surgery, treatment continuing until the animal's death. Those animals scheduled for the oral preparation were started on enterogastrone when the animal was able to return to the standard diet, or approximately one week postoperatively. Another group of animals received oral enterogastrone preoperatively for variable periods. The results are summarized in Table I.

RESULTS

The control series consisted of fifteen dogs which were maintained on the standard diet but received no other therapy after surgery. The average length of life for this group was eighty days. The minimum length was twenty-seven days while the maximum was 131 days. All control dogs died with ulcer. Of these, two thirds died from perforated ulcer including one dog with volvulus; the remainder died from ulcer hemorrhage.

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TABLE I

	Controls		
	15 dogs	No therapy	Average survival time: 80 days (27-131)
NUMBER OF DOGS	THERAPY	DOSAGE	AVERAGE SURVIVAL TIME
	Oral Enterogastrone		
6	Preop. (88-134 days)	8 Gm./day	108 days (56-224)
7	Postop.	8 Gm./day	96 days (40-147)
2	Postop.	16 Gm./day	73 days (44-103)
3	3 mo. preop. and 10-53 days postop.	8 Gm./day	104 days (96-122)
	Parenteral Enterogastrone		
15	(H) Postop.	200 mg./day	81 days (19-152)
4	I.M. Postop.	400 mg./day	87 days (19-134)

A group of eighteen dogs received the oral preparation. All dogs died with ulcer. Six animals were treated preoperatively, receiving 8 Gm. enterogastrone daily for periods of from 88 to 134 days. No correlation was found between the length of therapy and survival time in individual dogs. The average survival time was 108 days. Cause of death in five of these animals was perforated ulcer; the sixth died from bleeding ulcer.

In seven dogs treated postoperatively with 8 Gm. of oral enterogastrone daily, the average survival time was ninety-six days. Enterogastrone therapy was continued until death was imminent. All of these dogs died from perforated ulcer.

In the two dogs treated postoperatively with 16 Gm. of oral enterogastrone daily, the average survival time was seventy-three days. Again, therapy was continued until death. Both dogs died from perforated ulcer.

In three dogs treated three months preoperatively and ten to fifty-three days postoperatively with 8 Gm. of enterogastrone daily, the average survival time was 104 days. All dogs died from perforated ulcer.

A group of nineteen dogs received parenteral enterogastrone. Fifteen dogs received subcutaneous enterogastrone postoperatively in a dosage of 200 mg. daily. Therapy was continued until death. The average survival time was eighty-one days. All dogs died with ulcer. Eight died from perforated ulcer including one with chronic partial intestinal obstruction; three died from bleeding ulcer; four died with ulcer but without the immediate cause of death being apparent at autopsy.

Four dogs were treated postoperatively with intramuscular enterogastrone in a dosage of 400 mg. daily, the average survival time being eighty-seven days. Therapy was continued until death. All dogs died from perforated ulcer. The animal in this group which lived only nineteen days was found to be six weeks pregnant at autopsy.

Microscopic study of the ulcer specimens was done in approximately one-third of the treated animals. In no case was evidence of significant healing found.

SUMMARY

Nineteen Mann-Williamson dogs were treated postoperatively with parenteral enterogastrone without significant benefit, either as to survival time or prevention of ulcer formation. Eighteen dogs treated with oral enterogastrone showed slightly increased survival time over controls, but all ultimately developed ulcer.

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INFLUENCE OF SELF-ABSORPTION, VOLATILIZATION, AND DELIQUESCENCE IN COUNTING OF RADIOELEMENTS

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ERRORS in counting of radioelements may be due to absorption of radiation by the material in the sample counted, volatilization of the radioelement during preparation of samples for counting, or deliquescence, which varies the water content in a sample and therefore the absorption of radiation from the sample. The significance of these factors in radiobiology is often not fully appreciated. Their influence is especially likely to be reflected in radiobiology, since the chemical constitution of biologic fluids varies widely. This is particularly true of urine, which fluctuates considerably in pH, concentration, and chemical constitution, the latter changing both quantitatively and qualitatively from time to time. Obviously, when a radioelement of low energy is studied in successive samples of urine, it is important to ascertain whether any variations in counting rates are due to variations in self-absorption resulting from quantitative alterations in the solids of the urine or to variations in water content caused by differences in completeness of drying or deliquescence.

It will become evident from this report that drying of samples for counting by relatively mild heating may produce errors due to volatilization of the radioelement. Volatilization will vary not only with the degree and duration of heating but also with the chemical constitution of the biologic material in which the radioelement is found and with the radioelement employed.

Deliquescence may be of importance in counting biologic samples, particularly if they contain a large amount of solid material that is hygroscopic, such as is usually found in concentrated urine. Deliquescence, of course, varies with the temperature and humidity of the atmosphere of the laboratory as well as with the duration of exposure of the sample in a given atmosphere.

These and other factors have been studied for $Hg^{203, 205}$, Cl^{36}, Na^{22}, Na^{24}, K^{42}, I^{131}, and P^{32}† in distilled water, urine and plasma of man under the usual conditions of experimentation encountered in laboratories of radiobiology. This report is concerned with a summary of the results of these investigations and a discussion of their significance in tracer studies.

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*Life Insurance Medical Research Fund Postdoctorate Fellow (1948-1949), Tulane University School of Medicine and Charity Hospital of Louisiana.

†Except for the Na^{22}, the isotopes were obtained from the Oak Ridge National Laboratories. They were in the following chemical states: $Hg^{203, 205}$ as Mercuhydrin (methoxy-oxymercuripropylsuccinylurea with theophylline), Cl^{36} as HCl, Na^{22} as NaCl (obtained from Dr. Martin Kamen of Washington University, St. Louis, and Dr. M. A. Tuve at Carnegie Institute, Washington, D. C.), Na^{24} as Na_2CO_3, K^{42} as K_2CO_3, I^{131}, a mixture of I^{131} and I^{129}, and P^{32} as H_3PO_4. Radioactive Mercuhydrin was prepared through the courtesy of Dr. H. L. Daiell of Lakeside Laboratories, Milwaukee, Wis.

GENERAL METHODS AND MATERIALS

The influence of self-absorption, volatilization of the radioelement, and deliquescence was studied by preparing samples of distilled water, human urine, and blood bank or fresh heparinized human plasma containing various radioelements by means of the filter paper technique previously described.[1] Unless otherwise stated, all preparations contained 0.25 c.c. of the material measured and delivered by means of a micropipette already shown to be accurate and dependable.[2]

The samples were counted by means of a thin mica window Geiger counter. All counting rates were at least 1,000 counts per minute above background, which varied from 16 to 24 counts per minute. Counts were usually made for a five-minute period, and the level of counting was sufficiently high to be reproducible with a standard deviation of ±1 to 1.5 per cent for duplicate counts and still low enough to avoid significant errors due to coincidence counting. The performance of the scalers was checked several times a day by means of an impulse generator[3] and that of the counters and scalers combined was checked with a radium D and E standard obtained from the National Bureau of Standards. Corrections for background and physical decay were made, the counts reported throughout being counts per minute per 0.25 c.c. of material unless otherwise indicated. Although three separate counters and scalers were employed in these studies, each individual experiment was conducted on a single counter and scaler to avoid errors due to variations in sensitivity of the counters.

Weighings were carried out on a magnetically damped analytic balance accurate to ±0.1 milligram. This balance permitted rapid weighing so that satisfactory correlations between water content of a sample and its count could be made.

Sets of thirty slides each were prepared with water, urine, and plasma as the diluting fluids and were then counted. The necessary precautions mentioned throughout this report were employed. The mean over-all error, including all possible factors, was found to be 1.19 per cent and the standard deviation to be not greater than ±3.5 per cent for water preparations, ±2.1 per cent for urine, and ±4.0 per cent for plasma. These parameters are important in evaluation of the validity of individual counts.

RESULTS

Although there is considerable overlapping of the data, for purposes of discussion they are presented under the following headings: (1) self-absorption, (2) volatilization, and (3) deliquescence.

(1) Self-absorption.—The term self-absorption employed throughout this discussion denotes absorption of radiation by the entire sample, including water and all solid material, of which the isotope itself constitutes a part.

(a) Drying of the Filter Paper Disks: To determine the effectiveness of drying of the filter paper disks and the variations in water content associated with exposure of the samples to atmosphere of the laboratory, a group of twenty-nine disks and another of ten were prepared by delivering 0.25 c.c. of distilled water to each filter paper disk. The paper and metal disks were not cemented together.

Results are summarized by Tables I and II. The disks dried uniformly within two hours and changed insignificantly on further exposure to room air. Since significant fluctuations in water content are not likely to occur within a short period of time in the atmosphere of the laboratory, the special precautions required when a preparation is removed from an oven or desiccator were obviated. The influence of removing a filter paper disk from an oven is shown by Table II.

TABLE I. VARIATIONS WITH TIME IN WATER CONTENT OF 29 FILTER PAPER DISKS TO WHICH 0.25 GM. DISTILLED WATER WERE ADDED AND THEN ALLOWED TO DRY IN ROOM AIR

DURATION OF EXPOSURE OF DISKS IN ROOM AIR	MEAN REMAINING WEIGHT (GM.)	STANDARD DEVIATION (GM.)
1 min.	0.2456	±0.0008
4 hr.	0.0015	±0.0007
Overnight	0.0036	±0.0005

It is therefore important that the adequacy of drying of samples prepared for counting be determined experimentally for the materials employed and under the conditions of the investigation if satisfactory data are to be obtained. Obviously, small differences in water content will not influence significantly the counting rate of radioelements with high energies of radiation but may affect those of low energies.

TABLE II. VARIATIONS WITH TIME IN WATER CONTENT OF 10 FILTER PAPER DISKS TO WHICH 0.25 GM. WATER WAS ADDED AND THEN EXPOSED SUCCESSIVELY IN ROOM AIR AND AN OVEN

DURATION OF EXPOSURE	WATER CONTENT	
	MEAN (GM.)	RANGE (GM.)
A. Room air		
1. min.	0.2446	0.2436-0.2455
2. hr.	0.0012	0.0007-0.0024
4 hr.	0.0010	0.0008-0.0012
Overnight	0.0004	0.0000-0.0009
B. Heating for 15 min. at 82° C.	−0.0067*	−0.0043-0.0084

*This negative value represents the amount of water present in the "dry" filter paper which was driven off by heating.

(b) Self-absorption by Water: As previously indicated, the quantity of water in a filter paper disk may vary, but with standard drying conditions these variations are reduced considerably. It is also evident that under ordinary laboratory conditions there may be a disparity of a few milligrams in the water content of preparations, due in part to differences in completeness of drying of samples exposed to the atmosphere of the laboratory.

The influence of the water content in the filter paper disks was studied for $Hg^{203, 205}$, Cl^{36}, Na^{22}, Na^{24}, I^{131}, K^{42}, and P^{32}. Solutions of these isotopes were made in distilled water in a concentration which would produce a counting rate of approximately 1,000 CPM per 0.25 cubic centimeter. The total solids were low in these preparations. For preparations of the various isotopes in distilled water the absorping surface of the solids present varied between 0.0 mg. and 0.4 mg. per square centimeter; for preparations in samples of urine of varying specific gravities these variations ranged from 0.67 mg. to 1.67 mg. per square centimeter and for preparations in plasma from 1.79 to 3.2 mg. per square centimeter. The filter paper disks (Whatman No. 2) weighed 80 mg. and had an area of 8.39747 square centimeters.

Because of the rapidity with which weighing and counting had to be done to produce valid data for correlation, four slides were prepared for each isotope. Each slide was weighed, counted, and reweighed immediately after

pipetting and again after each of eight subsequent fifeen-minute periods of drying in room air. The mean count considered as 100 per cent was obtained after stability of counting or a plateau value had been reached (Fig. 1), and all counts are expressed as percentage of this mean.

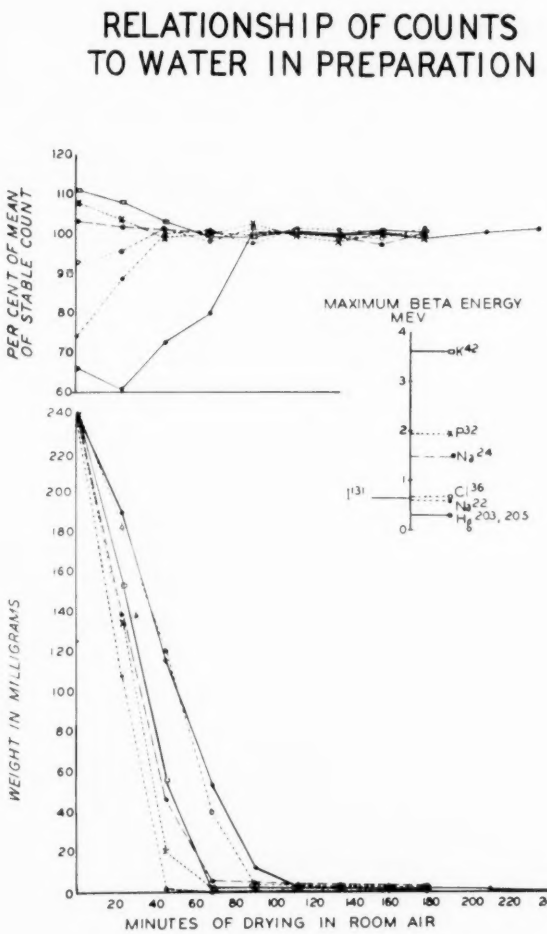

Fig. 1.—Time-course of the relationship of the rate of counting (upper curves) to water content (lower curves) of filter paper disks. As the preparations dry and become stabilized in weight, so do the counts. The radioelements with a considerable portion of the spectrum of radiation at the low levels of energy ($Hg^{203, 205}$, Na^{22}, and Cl^{36}) exhibited a high percentage of absorption of radiation by the water in the preparation. Those radioelements with high energies of radiation (K^{42}, Na^{24}, and P^{32}) had higher rates of counting when the preparations contained the greatest amount of water. These relationships are seen to exist throughout all the figures to follow. Insert shows the maximum β-ray energies of the isotopes studied.

Results are summarized by Fig. 1. It is evident from these data that there is self-absorption by the water in the preparation of $Hg^{203, 205}$, Na^{22}, and Cl^{36}. Isotopes of high energies of radiation, such as K^{42}, Na^{24}, and P^{32}, had no loss due to self-absorption. Details of correlation of the water content of the preparation with the level of counting are shown in Fig. 1. No attempt was

made to explain the higher counts for K^{42}, Na^{24}, and P^{32} when the preparations were wet than when they were dry, but this observation appeared to be directly related to the β energy of the isotope.

Because of the well-known importance of volatilization of iodine from preparations made for counting, the I^{131} was considered separately. Filter paper disks were prepared with I^{131} in distilled water and also in a special diluent consisting of 1.02M NaOH, 0.0015M KI, and 0.005M $NaHSO_3$. To one set of each of these preparations, 0.1 mg. $AgNO_3$ was added. The slides were weighed and counted at fifteen-minute intervals, as with the other radioelements.

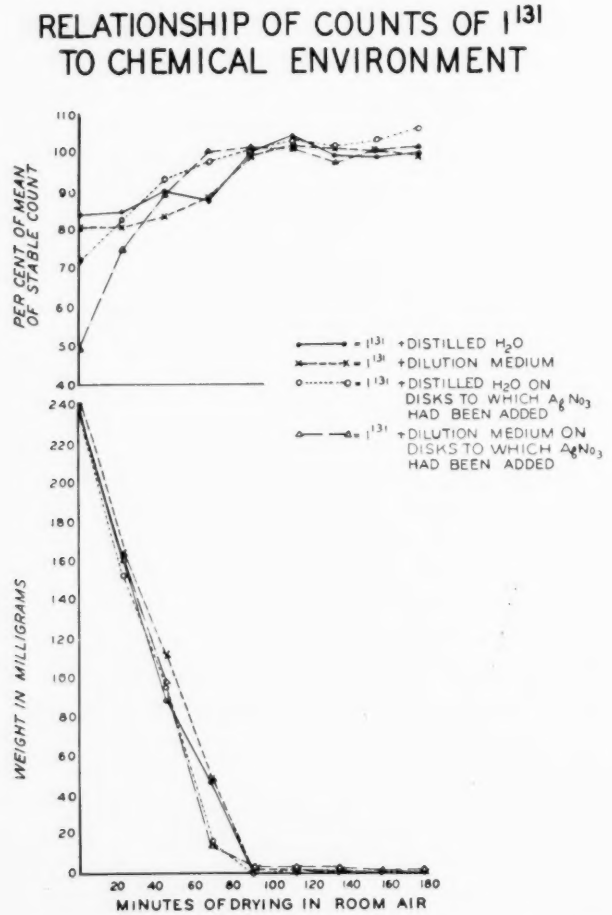
RELATIONSHIP OF COUNTS OF I131
TO CHEMICAL ENVIRONMENT
PER CENT OF MEAN OF STABLE COUNT
WEIGHT IN MILLIGRAMS
MINUTES OF DRYING IN ROOM AIR
I131 +DISTILLED H2O
I131 +DILUTION MEDIUM
I131 +DISTILLED H2O ON DISKS TO WHICH AgNo3 HAD BEEN ADDED
I131 +DILUTION MEDIUM ON DISKS TO WHICH AgNo3 HAD BEEN ADDED

Fig. 2.—Time-course of the relationship of rate of counting to chemical environment and water content of preparations of I^{131}.

Results are summarized by Fig. 2. It is evident that the method of chemical preparation influenced the results, especially the absolute values when the preparations were wet. The water in the preparation also influenced the rate of counting through the effects of self-absorption (Fig. 2).

Comparison of preparations of the various isotopes in distilled water, urine, and plasma revealed no significant differences when the filter paper technique was used and the slides were dried at room temperature. On the other hand, when the same volumes of the same solution of these radioisotopes were dried on metal disks without filter paper, considerable differences between urine, plasma, and distilled water were noted (Table III). The counts of the preparations in water were uniformly higher than those in urine, which, in turn, tended to be higher than those in plasma. In addition, the distilled water preparations on metal disks were considerably higher than those of the same solution in equal volume on filter paper. These differences were most noticeable when low energy radioelements were employed. Two factors appear to be responsible for the discrepancies: (1) the variation in geometric relationship due to position of the sample when these three different solutions were dried on the metal disks and (2) self-absorption by the filter paper of back scatter emanation by the particles of the sample. Deliquescence enhanced the latter and will be discussed later.

TABLE III. COMPARISON OF MEAN OBSERVED COUNTS PER FIVE MINUTES OF PREPARATIONS MADE ON METAL DISKS WITH PREPARATION OF THE SAME VOLUME OF THE SAME SOLUTION ON FILTER PAPER SLIDES; BOTH GROUPS DRIED IN THE SAME MANNER

	WATER PREPARATION CP5′	URINE PREPARATION CP5′	PLASMA PREPARATION CP5′
$Hg^{203, 205}$			
Metal disks	19,587	11,990	9,458
Filter paper on metal disk	6,975	6,682	6,797
Cl^{36}			
Metal disks	10,765	11,410	7,590
Filter paper on metal disk	6,697	6,747	6,865
Na^{22}			
Metal disks	9,846	8,785	4,423
Filter paper on metal disk	4,938	4,975	4,999
K^{42}			
Metal disks	20,717	22,808	21,634
Filter paper on metal disk	15,231	13,856	14,624

(2) Volatilization.—In the standardization of the counting technique it was observed that the radioelements may volatilize during preparation, especially if heating is employed to hasten drying. To evaluate this phenomenon more carefully, filter paper disks were prepared as described previously for the same radioelements except Na^{24} and P^{32}, which were not available at the time, although the experiments with Na^{22} sufficed to clarify radiosodium. A series of four or eight disks was prepared for each isotope. The water content was determined by weighing immediately after the preparations were made. They were then counted and reweighed. The slides were then placed in an oven at 84° ± 2° C. for ten minutes, reweighed, counted, and weighed again. The time required for each weighing varied from thirty to sixty seconds. This was repeated three times successively. After the third heating with weighing and counting, the disks were exposed to the room air for one and twenty-four hours or longer, as noted by Fig. 3, and then were weighed and counted again.

For controls, disks were prepared in the same manner from the same solution and were dried by exposing them to the laboratory atmosphere for four hours. The mean count of these disks constituted the respective 100 per cent values.

Since heating of the slides would result in curling of the filter papers, thereby affecting the geometric pattern and consequently the counting rates, the filter papers were cemented to the metal disks before addition of the solu-

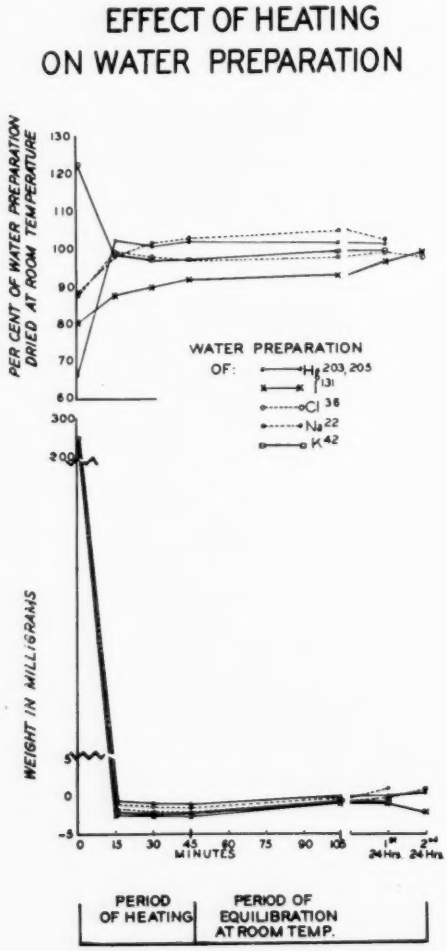

Fig. 3.—Time-course of the relationship of rate of counting to water content of the filter paper disks as influenced by mild heating of $Hg^{203, 205}$, Cl^{36}, I^{131}, Na^{22}, and K^{42} dissolved in distilled water. The 100 per cent value was obtained from the mean count observed for the preparations dried at room temperature.

tions. It was demonstrated that there was no significant difference in results when the filter papers were cemented to the metal disks before the addition of solution rather than after the solution was dried on the filter paper.

Results are summarized by Figs. 3, 4, and 5. The results with disks prepared with water solution were the same for the slides dried by heating as for

those exposed three to four hours at room temperature except for I^{131}. Iodine volatilized from the heated preparation of I^{131} in distilled water, urine and plasma in varying amounts, being most noticeable with distilled water and least noticeable with the plasma preparation. It should be emphasized that iodine was likewise lost from unheated preparation but to a lesser degree. Because of the loss at room temperature, volatilization after heating tends to be less apparent than for mercury in urine (Fig. 4). In the latter there was a decided decline

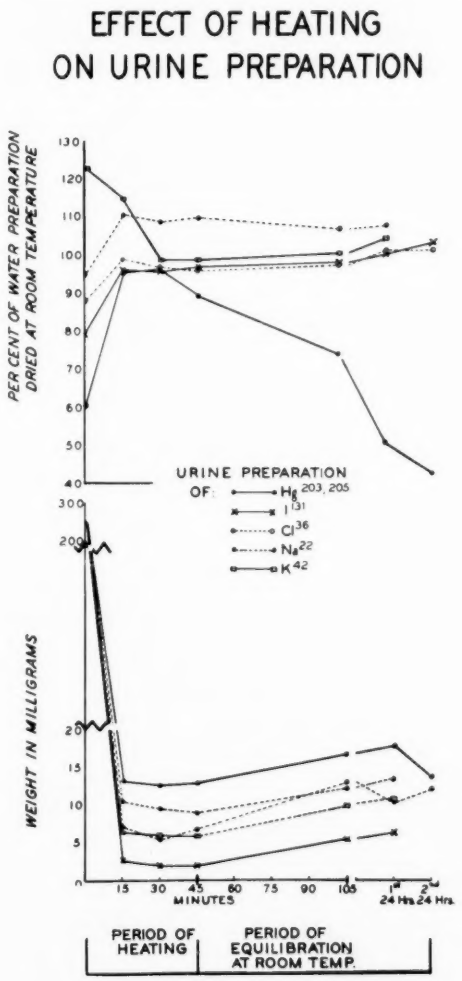

Fig. 4.—Time-course of the relationship of rate of counting to water content of the filter paper disks as influenced by mild heating of $Hg^{203, 205}$, Cl^{36}, I^{131}, Na^{22}, and K^{42} in human urine.

in radioactivity, which can only be attributed to loss of the mercury due to volatilization of the $Hg^{203, 205}$ and not to self-absorption, as the weight of water present was insignificant when the counts were lowest. This volatilization of mercury from heated normal urine slides was observed repeatedly in both in vitro and in vivo studies.

Fig. 6 shows the effects of heating preparations of mercury in water, urine, and serum for varying periods of time over a temperature range of 45° to 200° C. The influence of the chemical environment is apparent; the mercury volatilized from urine slides at a considerably lower temperature than from water and serum. At a given temperature the times of exposure studied

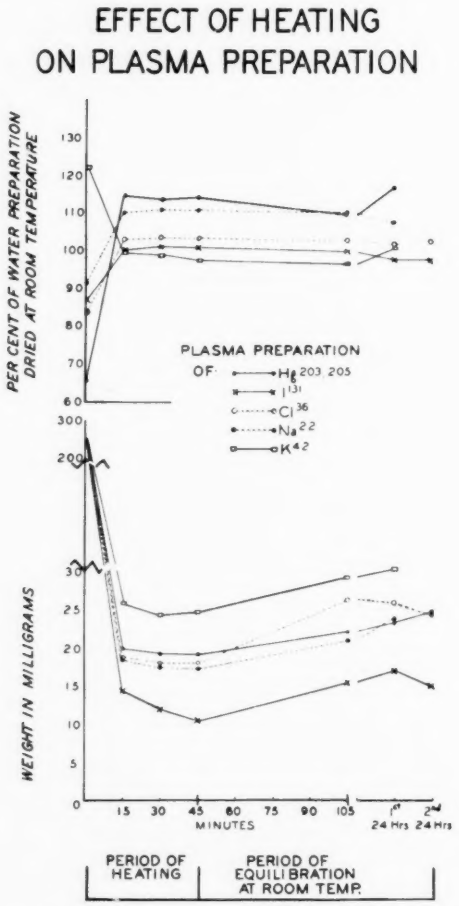

Fig. 5.—Time-course of the relationship of rate of counting to water content of the filter paper disks as influenced by mild heating of $Hg^{203, 205}$ Cl^{36}, I^{131}, Na^{22}, and K^{42} in human plasma.

(twenty, forty, and sixty minutes) did not influence the results greatly. No attempt was made to define the factor or factors present in urine which effect this result. Studies of preparations made on glass and plastic disks showed that amalgamation of the mercury was not a factor concerned with the decline in counting level. Furthermore, counts of the metal disks with the filter paper removed registered background. In these groups of experiments, as in the previous ones, the level of counting was highest for the K^{42} when the preparation contained the greatest amount of water.

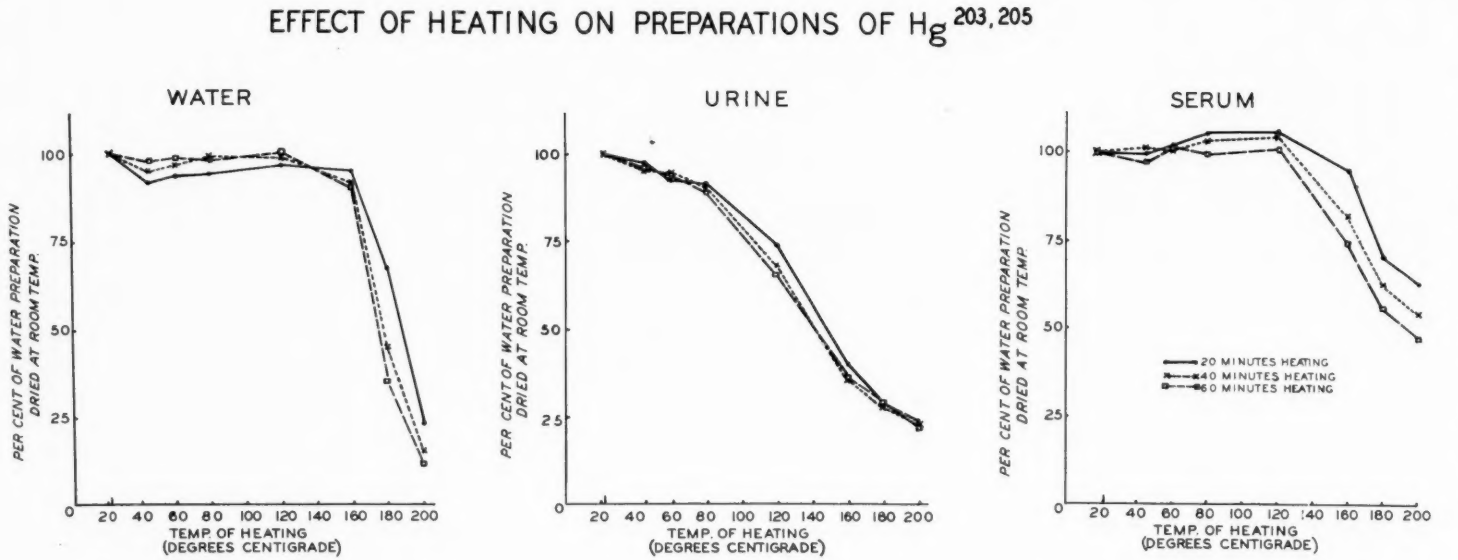

Fig. 6.—Influence of heating upon rate of counting of preparations of $Hg^{203, 205}$ in water, urine, and serum. Mercury began to volatilize from the water and serum slides at a temperature of 120° C., whereas loss of mercury from urine began at 45° C. Duration of heating was not as important as the temperature at which the preparation was heated and the chemical environment of the mercury.

Further evidence of volatilization of $Hg^{203, 205}$ from preparations made for counting was obtained from the following observations.

(a) Disks containing $Hg^{203, 205}$, followed several weeks for physical decay, showed a rate of decline which was greater than that expected from published studies of its physical half-life and more rapid than that obtained from the same material sealed in a glass ampule. The most rapid decay in counts was found for disks prepared with human urine as a diluent, the next most rapid when water was used, and the least rapid with human serum.

(b) The rate of decline in counts in a sample increased proportionately with the increase in temperature of heating or duration of heating. The influence of temperature on counting varied with the nature of the chemical environment of the mercury compound, being more rapid in urine, feces, and lactose, for example, than in water, serum, or plasma.

(c) The rate of decline in counts was more rapid in a vacuum than in room air at the same temperature.

(d) Nonradioactive filter paper disks placed in a closed container with other disks to which radioactive mercury had been added progressively acquired radioactivity. When filter paper disks containing radiomercury in different concentrations were placed in the same closed container at room temperature, those preparations with the lower counts gained counts and those with the higher lost counts.

(e) A previous publication indicated the possible separation of radiomercury from radiosodium in the same filter paper disk by means of heating and volatilizing the mercury.[4]

In Fig. 5 the counts of the heated slides of plasma solutions of the various radioelements are expressed as percentage of unheated water slides. Here, instead of decreasing because of volatilization, the counting of heated slides of mercury, sodium, and chlorine was approximately 15, 10, and 3 per cent respectively higher than the unheated water slide.

As has been stated previously, unheated water and plasma slides do not differ significantly. When plasma slides of mercury dried by heating were compared with plasma slides dried at room temperature four hours and then heated in the same manner, the former still counted about 15 per cent higher than the latter. Finally, when plasma slides dried by heating were equilibrated four hours at room temperature and recounted, the same differences were observed. It must be concluded then that the process of drying plasma slides by heating at this temperature (84° C.) alters the counting rate significantly. In Fig. 4 it is noted that the heated preparation of Na^{22} in urine counted 10 per cent higher than unheated water preparations. Here, as with the problem of self-absorption, isotopes of low energy are affected whereas those of high energy are not.

In order to study the role of heating in the previously mentioned variation of plasma preparation of $Hg^{203, 205}$, a set of slides was prepared and dried at room temperature and another set was prepared from the same solution and dried by heating for fifteen minutes at 84° C. Control distilled water preparations were made. In half of each set the filter papers were cemented to the

metal disks in the usual manner. In the remaining slides the filter papers were inverted and cemented to the disks. All slides were counted for five minutes on the same counter. Fig. 7 summarizes these data. Here one notes that the heated plasma slides prepared and counted in the usual manner are 13.9 per cent higher than unheated plasma slides. The second set of slides in which the filter papers were inverted (i.e., "face down") and cemented to the desk counted 14.3 per cent lower than "face up" slides when both were heated. Unheated plasma and water preparations fell 8.6 and 7.4 per cent respectively with inversion of the filter paper. Thus it seems that heating of

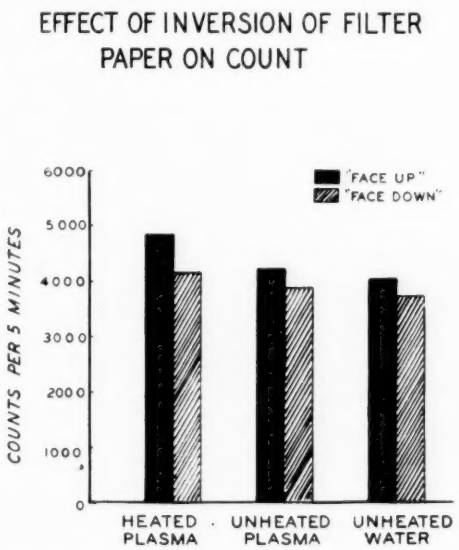

Fig. 7.—Effect of inversion of the filter paper on the metal disk after drying of the preparation of $Hg^{203, 205}$ at 84° C. and at room temperature. Heated plasma preparations with filter papers pasted to metal disks in the usual manner (i.e. "face up") counted 14.3 per cent higher than similarly prepared slides with the filter papers pasted "face down" on metal disks. Slides of unheated plasma and distilled water exhibited differences of 8.6 and 7.4 per cent respectively between "face up" and "face down" preparations.

the plasma slides alters the geometric pattern of placement of plasma containing mercury in the strata of the filter paper. These observations indicate a need for caution in calculating absorbing thickness of a preparation made for counting.

The P^{32}, Na^{22}, and K^{42} were studied at the same temperatures as the Cl^{36} and $Hg^{203, 205}$ and showed no evidence of volatilization. A temperature of 220° C. was not exceeded in these studies. Fig. 8 shows the effect of heating preparations of Cl^{36} in distilled water over a temperature range of 45° to 200° C. for varying lengths of time. As in Fig. 6 for $Hg^{203, 205}$, the duration of heating did not influence the results as much as the temperature at which the preparations were heated.

(3) Deliquescence.—The influence of deliquescence is revealed in Figs. 4 and 5. These two illustrations show that the filter paper disks gained weight with standing in the laboratory after heating. Although no detailed studies

such as simultaneous weighing of the preparations with counting were made, it was observed during the development of techniques that when preparations of urine containing large quantities of solids were placed on the metal disks without filter paper and were dried, deliquescence often became so conspicuous that the solids were redissolved by a small pool of the water accumulated from the atmosphere. The accumulated water would, of course, absorb low energy radiation (especially back scatter) from the preparations. Such extensive wetting of the preparations as a result of deliquescence has never been encountered with the filter paper technique.

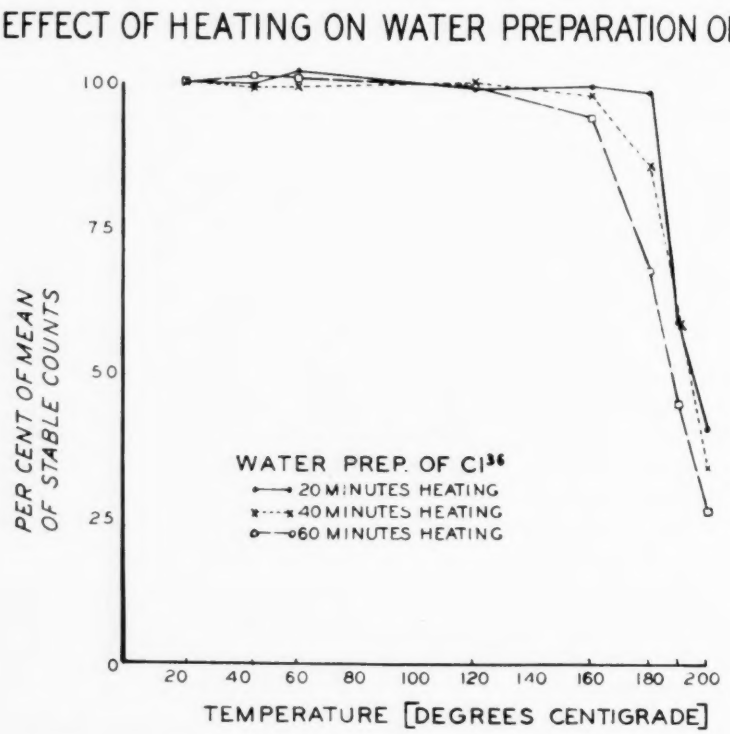

Fig. 8.—Influence of heating upon rate of counting of Cl^{36} in distilled water prepared on filter paper disks. When a temperature of 160° C. was reached, loss of radiochloride occurred by volatilization and increased as the temperature rose or duration of exposure to temperature lengthened.

In order to investigate this observation in greater detail, nine slides containing $Hg^{203, 205}$ in human urine were prepared by dropping 0.2 c.c. in the center of the metal disks and then drying them by exposure to 63° C. for twenty-five minutes. The slides were counted and then were exposed to the room air overnight. The next day deliquescence had produced a pool of water in the area previously occupied by the urine which had been dried. After the slides were counted again, they were placed in the oven at 63° C. for twenty-five minutes and were then recounted. The results are summarized by Fig. 9. The mean count after the initial drying was about 23 per cent higher than the mean count on the next day after deliquescence had occurred. Redrying

resulted in a rise of about 7 per cent in the counting rate. The difference in the counting rates after the initial and second periods of drying with heat is due to volatilization, which is supported by the experiments summarized by Figs. 4 and 6. The difference between the second and third counts shown in Fig. 9 represents the minimum effect of deliquescence. Any additional effect of deliquescence was obscured by volatilization.

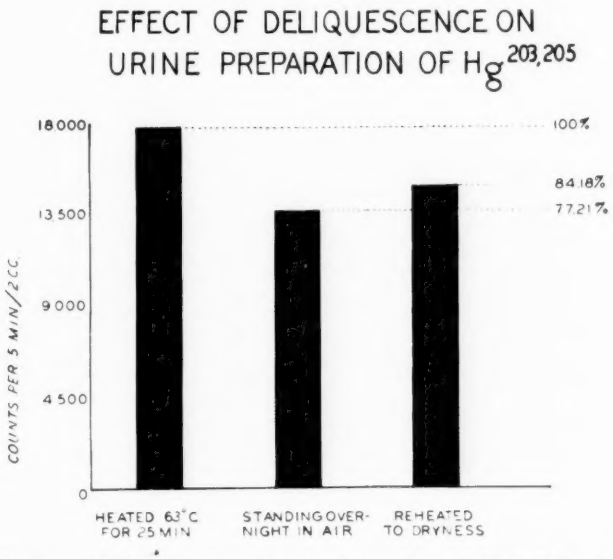

Fig. 9.—Influence of deliquescence on rate of counting of $Hg^{203, 205}$ in human urine prepared on ten metal disks without the use of filter paper. The difference in counts reflects the effect of both volatilization and deliquescence. Since volatilization was also occurring, the difference between the second and third counts represents the minimum effect of deliquescence.

It is evident from the other illustrations showing studies with the same radiomercury compound that deliquescence did not introduce a significant error when the filter paper disk technique was used. This is one reason why this technique is advocated in certain types of tracer studies in radiobiology.

GENERAL DISCUSSION

The data presented show that under certain circumstances self-absorption, volatilization, and deliquescence may influence counting rates significantly. Their importance in any study employing radioelements must be properly evaluated. Self-absorption is particularly likely to be important in tracer studies in biology if radioelements with relatively low energies of radiation are being employed and if the solid content of the biologic fluid varies considerably. The latter would be particularly true of urine. This phenomenon is most likely to be encountered with the gas flow type of counter, which is sensitive to the low energy level of the spectrum of radiation. It has been found that most uniform results are obtained when the filter paper disks are dried in room air. The only disadvantage of this might be the time required for drying. If heating is employed, the influence of variations in degrees of

drying, volatilization, and deliquescence must be carefully evaluated before such a method or technique can be accepted as satisfactory.

Volatilization of the radioelement from the preparations may be an extremely significant variable under certain experimental conditions, particularly when high temperatures are employed at some stage of the procedure.

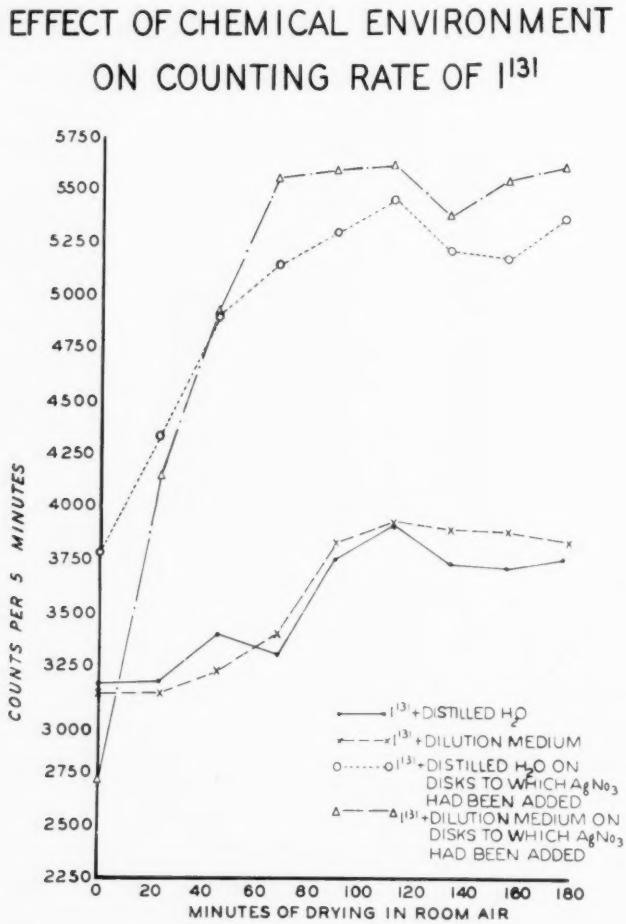

Fig. 10.—Time-course of rate of counting of I^{131} prepared on filter paper disks as influenced by chemical environment. The presence of silver nitrate definitely reduces the loss of the radioiodine by volatilization.

This is certainly true for iodine and mercury at even ordinary room temperature and at only moderately elevated temperature for chlorine. It is possible that some of the experiments in which chemical methods of analysis associated with ashing are employed in the preparation of samples may yield erroneous results because of loss of the elements by volatilization. The use of isotopes for pilot experiments to check procedures for losses by volatilization has been discussed elsewhere in greater detail for tracer studies with Cl^{36} in dogs.[5]

It is possible, as is shown for iodine (Fig. 10), that special chemical treatment of the material may be necessary to prevent volatilization. It is also evident from the observations with radiomercury that volatilization may vary with the chemical environment of the material (Figs. 3, 4, 5 and 6). It would be inaccurate, therefore, to interpret absence of volatilization in one biologic fluid as indicative of its absence in another.

Deliquescence may influence the counting rate of radioelements with radiations of low energy. This is essentially the same problem discussed previously for uniform drying to avoid variations in self-absorption by the water in the preparation. The problem of deliquescence is concerned primarily with maintaining a uniform state of dryness, which was satisfactorily accomplished by the use of the filter paper disk technique with drying in room air.

SUMMARY

A study of the behavior of $Hg^{203, 205}$, Cl^{36}, Na^{22}, Na^{24}, K^{42}, P^{32} and I^{131} in water, human urine, and human plasma, in vitro and in vivo, indicated that self-absorption, volatilization, and deliquescence offer special problems in radiobiology. Although these factors behave exactly as would be expected, they may be overlooked because of some of the peculiar experimental conditions encountered in biologic investigations. A correlation of gravimetric and radiometric determinations of preparations of the previously mentioned isotopes in water, urine, and plasma on filter paper disks after drying them at various temperatures revealed that to control these factors careful attention should be paid to the chemical environment and, more important, the method and temperature of drying the biologic materials for counting.

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SUCROSE ABSORPTION IN SPRUE

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THE oral glucose tolerance test has been used as a method for detecting impaired carbohydrate absorption in sprue. One group of investigators[1] studied the absorption of both fructose and glucose in patients with sprue. From their data they concluded that the glucose absorption was much impaired, but that fructose absorption was unaffected. Their explanation for this differential phenomenon was based on the theory that glucose absorption depends upon phosphorylation, and that the malabsorption of glucose in sprue results from nonphosphorylation. Fructose, it was stated, passes across the gut membrane by a process of simple diffusion.

If it could be established that patients with sprue are capable of absorbing fructose in a normal fashion, then the problem of dietary management and weight restoration might become more simplified. The present study deals with investigations of the simultaneous absorption of fructose and glucose in sprue and in normal patients.

METHODS

Ten normal, healthy adults were first studied. All had been fasting for at least a twelve-hour period. After obtaining a fasting blood sample, 100 Gm. of sucrose dissolved in 300 ml. of water were given by mouth rapidly. Samples of venous blood were drawn at intervals during the next three hours. Glucose and fructose values in the blood samples were promptly determined. Benedict's macromethod[2] was used for blood glucose determinations. The fructose values were determined by the modified skatole color reaction method of Reinecke.[3]

The same procedure was applied to seven adult patients with sprue. All of this group were selected on the basis of clinical and laboratory evidence typical of the sprue syndrome.

RESULTS

The original data are presented in Table I. The form of curves derived from these data, relating blood glucose values and blood fructose values to time, is indicated in Figs. 1 and 2. A heavy line passes through the mean values.

In *normal control patients,* the blood glucose rose promptly following the ingestion of sucrose, then began to fall rapidly. In these same normal patients, the fructose values rose more slowly, but at the end of three hours had returned to fasting levels.

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TABLE I. SUCROSE TOLERANCE TESTS ON NORMAL PATIENTS AND PATIENTS WITH SPRUE (BLOOD LEVELS AT MINUTES INDICATED AFTER INGESTION OF 100 GM. OF SUCROSE)

CASE	CLINICAL MATERIAL	GLUCOSE (MG. PER 100 ML. BLOOD) 0′	30′	60′	120′		FRUCTOSE (MG. PER 100 ML. BLOOD) 0′	30′	60′	120′	180′
1	Normal patients	93	154	125	91	82	2.0	7.5	8.0	8.0	2.8
2		100	130	143	123	90	2.0	8.0	10.0	4.7	2.6
3		92	158	200	103	66	2.1	5.0	10.6	6.9	2.5
4		117	188	190	135	84	1.9	5.6	7.1	6.9	2.2
5		90	142	127	95	80	2.9	7.9	8.7	4.1	2.3
6		90	189	149	108	76	2.1	8.7	8.5	8.3	3.1
7		108	200	190	128	68	2.3	7.8	9.0	5.3	3.1
8		91	156	156	105	62	3.1	8.2	8.7	7.1	2.1
9		90	148	115	87	70	1.7	6.6	9.1	5.3	3.1
10		87	182	182	108	67	1.5	7.5	9.3	5.3	1.7
Mean		95.8	164.7	157.7	108.5	74.5	2.7	7.3	8.9	6.2	2.5
Stand. dev.		9.6	23.3	30.9	15.9	9.2	0.49	1.18	0.97	1.43	0.48
1	Sprue	86	108	138	81	78	1.3	4.9	6.9	5.1	6.0
2		88	125	120	105	90	1.5	5.6	6.9	6.3	6.2
3		102	156	115	100	86	1.9	5.0	5.6	7.0	5.8
4		98	102	112	96	98	2.5	3.0	4.4	6.0	6.6
5		90	138	118	94	71	2.0	7.2	8.1	8.7	4.0
6		114	125	143	126	85	4.6	4.6	8.7	10.3	7.9
7		104	115	100	95	85	1.9	6.2	6.9	7.9	4.4
Mean		97.4	124.1	120.8	99.6	84.7	2.2	5.2	6.8	7.3	5.8
Stand. dev.		10.1	18.5	14.9	13.8	8.6	1.21	1.32	1.44	1.77	1.32

In *patients with sprue*, the blood glucose rose fairly promptly after ingestion of sucrose, but the mean rise was much lower than normal. The blood fructose rose more slowly, was sustained, and did not fall to fasting levels at the end of three hours.

For statistical purposes the standard deviation of the means at each time interval was computed. The means and the standard deviations of the means at each point are presented in Table I. By the use of the *t* test, these figures showed that the mean fructose values in the normal patients were significantly different from those of patients with sprue at thirty minutes, one hour, and three hours after ingestion of the sugar. In normal patients, the fructose values reached higher levels at thirty and sixty minutes than in sprue. Also, the values for fructose in normal patients returned to fasting levels at three hours, whereas in sprue the fructose values after three hours were still significantly elevated. There was no significant difference between normal patients and those with sprue at the two-hour period, but the trend of fructose values in the normal patient was on the way down, whereas this trend was not noted in the group with sprue. (See Figs. 1 and 2.)

In the case of glucose, there was such a range of variations in individual values that no significant differences of the means between the normal and the patients with sprue could be demonstrated at any single time interval. However, there was an apparent difference in mean glucose values as noted by the form of the curves in Figs. 1 and 2.

DISCUSSION

The glucose absorption curves in the group of normal patients were within normal limits, whereas the absorption of glucose in the patients with sprue differed from the normals by rarely reaching the upper range of normal values.

The blood fructose level in normal patients should reach a maximum level of approximately 10 mg. per 100 ml. of blood in one hour following the ingestion of sucrose.[4] The fructose absorption curves in our normal patients were within normal limits. The fructose absorption in our patients with sprue was of lower magnitude than in the normal subjects, and furthermore, the blood fructose in sprue did not return to fasting levels at the end of three hours as in the normal patients.

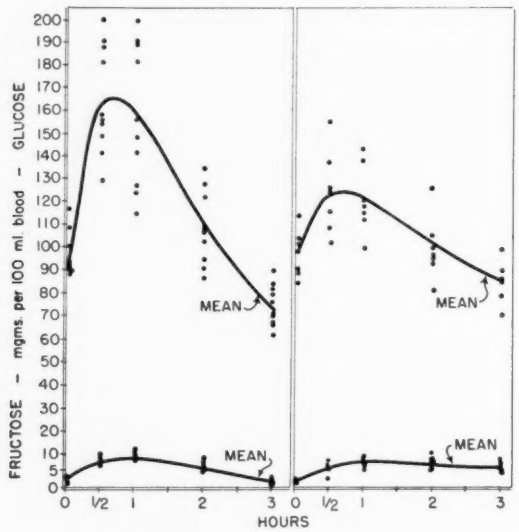

Fig. 1. Fig. 2.

Fig. 1.—Sucrose tolerance curves in ten normal patients.
Fig. 2.—Sucrose tolerance curves in seven patients with sprue.

It is of chief interest to find an explanation for the difference in the manner in which the fructose is handled by the patient with sprue and the normal patient. It has been determined[5] that the rates at which various sugars are converted into liver glycogen are not the same. The blood sugar curve is the resultant of several metabolic processes of which absorption is only one, all going on simultaneously at an unknown rate. In our group of patients with sprue, the absorption of both fructose and glucose was defective in most instances. It would seem that the sustained rise of fructose by the patient with sprue is abnormal, the explanation of which is not clearly understood.

SUMMARY

Results of the sucrose tolerance test in ten normal subjects and seven patients with sprue are described.

In normal patients the fructose values at thirty and sixty minutes reached higher levels than were attained by sprue at the same time intervals.

Also, the fructose values in normal patients returned to fasting levels after three hours, in contrast to a sustained elevation by the patients with sprue at this period.

The shape of the glucose absorption curve in sprue differs from that seen in normal subjects, because the upper range of normal values are found only rarely.

It appears that the defective absorption of carbohydrate in sprue is not one of selective difference as to the hexoses used in this experiment.

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LABORATORY METHODS

A METHOD OF PREPARING BIOLOGIC FLUIDS FOR COUNTING OF RADIOELEMENTS

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BECAUSE the method of preparing samples of biologic fluids containing radioelements employed in tracer studies in this laboratory has proved to be simple, accurate, inexpensive, and dependable in many experiments, its description was considered advisable. The procedure insured reproducibility in large part because of more even distribution of the sample and, therefore, more constant geometric relationship. It is applicable not only to studies in experimental animals, where danger to the animal from radiation does not limit the dosage of radiation, but also to studies in man, in whom injury from radiation is particularly hazardous and the dosage is therefore restricted.

Procedure.—The procedure consists in delivering a known volume, usually 0.25 or 0.30 c.c., of the biologic fluid under study onto filter paper disks* by means of a micropipette.[1] These are placed upon flat, tin-coated metal disks† approximately 37 mm. in diameter and 0.305 mm. thick. A lip of about 0.1 mm. is produced on the metal disks during their preparation, which prevents spillage of fluid should careless tilting or excessive delivery of a sample occur. The diameter of the filter paper should be less than that of the window of the counter, and the center of the filter paper should coincide with the center of the window of the counter. To insure a proper and constant geometric relationship, brass or aluminum receivers were machined to hold the disks in position during counting (Fig. 1,*A*). Circular filter paper and metal disks were used for the mica window counters with circular windows (Fig. 1,*B*, *C*, and *D*), but uniform, rectangular sheets of filter paper, mounted upon glass microscope slides, were employed in a similar fashion and with equal success for the long glass window counters (Fig. 1,*E*, *F*, *G*, and *H*). A stack of metal disks and of filter paper placed in a Lucite dispenser suspended in the laboratory makes them readily available.

In use, a single filter paper disk is placed in the center of a metal disk (Fig. 1,*D*), and the material for counting is dropped from the micropipette onto the filter paper at different points to insure uniform spreading. For disks of the size indicated, 0.25 to 0.30 c.c. of any biologic fluid will uniformly saturate the filter paper without a tendency to spread beyond, onto the edges of the metal disk.

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*The filter paper disks, each approximately 33 mm. in diameter, were punched by a stationery dealer from large sheets of Whatman No. 2 filter paper.

†The metal disks were obtained from the American Can Company at $7.50 per 100 pounds or about 15,000 disks.

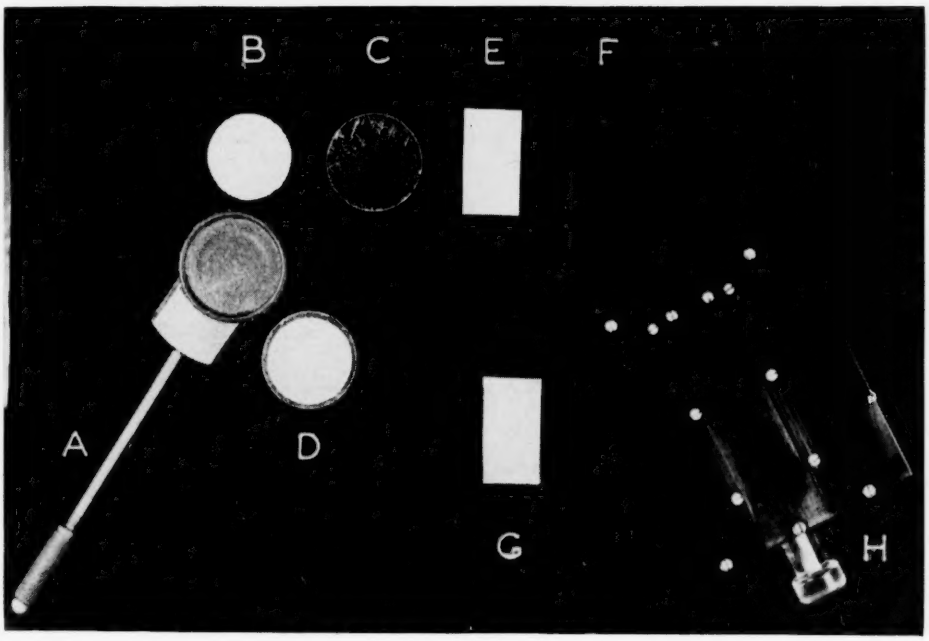

Fig. 1.—The materials used in preparing the samples of biologic material for counting.
A, The receiver machined to hold the disks in place under the counter.
B, A filter paper disk.
C, A tin-coated metal disk.
D, The filter paper cemented in place on the metal disk.
E, A rectangular filter paper sheet for use with glass window counters.
F, A glass microscope slide.
G, A filter paper sheet cemented onto a glass slide.
H, A bakelite tray modified to ensure a constant slide-counter relationship.

After the sample has been dropped upon the filter paper disks, the preparation is set aside to dry. If heating in an oven is employed to hasten drying, care should be taken to ascertain whether heating produces any loss of the radioelement by volatilization. This special problem is discussed in detail elsewhere.[2] It is necessary that all samples be dried uniformly in order to avoid errors due to absorption of radiation by water present in the preparation. Uniform, adequate drying may be obtained in room air, provided the humidity of the atmosphere is not too high. Errors due to deliquescence may occur if concentrated solutions are being studied; this is particularly true if the room air is humid. The influence of drying and deliquescence must be evaluated properly regardless of the method employed in preparing samples for counting. Deliquescence did not introduce errors in any of the biologic fluids such as plasma, serum, whole blood, urine, edema fluid, diluted feces, tears, or gastric juice of man, which were studied by means of filter paper disks, but did offer difficulties, especially with concentrated urine, when filter paper was not used.

After the preparation is dried, the surface of the metal disk and the back of the filter paper disk are covered with a thin film of rubber cement, which is allowed to dry. The surfaces of the metal and filter paper disks covered by rubber cement are brought together, and the paper disk is pressed smoothly in place. No significant error is produced by coating the tin disks with the rubber cement, since there is no demonstrable adhesion of radioelements to the metal disks after drying is completed. Once the sample has dried, touching the disks of filter paper will not introduce any demonstrable errors or remove any measurable amounts of the radioelement. Since many disks are usually prepared at one time, they may be stacked and a weight placed thereon to assist in uniform and smooth cementing while other preparations are being counted.

Each preparation may be easily and permanently labeled by writing a code number or label upon the white filter paper disk with a No. 2 lead pencil before delivering the sample of biologic fluid upon it with the micropipette. Because these disks are so inexpensive, the necessity of cleaning them for re-use is eliminated and they may be either discarded or filed in envelopes, small boxes, or stacks for future reference or for checking of counts if the physical half-life of the radioelement permits.

RESULTS

Evaluation of Errors.—The accuracy of the procedure of preparing samples for counting was tested from many aspects. No measurable errors were observed to be produced by the use of the thin coat of cement or by touching and gentle handling of the filter paper disks, once they were dried, either during

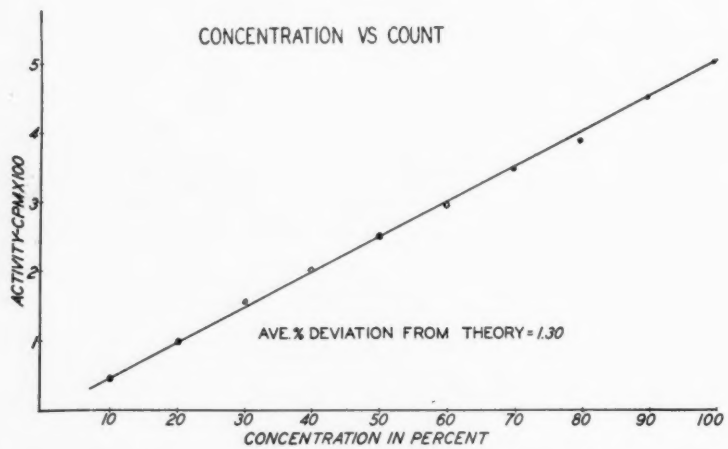

Fig. 2.—Relation of concentration of Na^{22} to counts. The line is the theoretically perfect line and the points are the observed values. The rate of counting selected was that usually encountered in the body fluids of man under experimental conditions with "safe" doses of radioactivity.

the process of cementing the paper and metal disks together or thereafter. The influence of centering of the paper disks upon the metal ones was studied, and no errors were detected as a result of failure to center the paper disk precisely; nor was an error produced when disks were stacked, once the filter papers were dried.

To evaluate the over-all error involved in preparing disks, the reproducibility in preparation of disks for counting was studied. A radioactive element was added to water, plasma, and urine in vitro and serum, plasma, urine, and ascitic fluid of subjects who had received a previous injection of a radioelement were also used to prepare thirty or more consecutive disks from each test solution. The same procedure was employed for $Hg^{203-205}$, Cl^{36}, Na^{24}, Na^{22}, I^{131}, P^{32}, and K^{42}. The mean error was found to be 0.77 per cent, with a standard error of $\pm$ 0.39 per cent. With elements as volatile as mercury or iodine, certain additional precautions are necessary to avoid errors due to losses of the radioelements by volatilization.[2]

The influence of variations in concentration of the radioelement in a sample upon the accuracy of counting of samples was tested by preparing ten separate dilutions varying by 10 per cent each from 10 to 100 per cent and by preparing thirty disks for each dilution. The results shown in Fig. 2 indicate a mean error of only 1.30 per cent, which is relatively small when it is realized that the counting rate varied from 40 to 500 counts per minute above background.

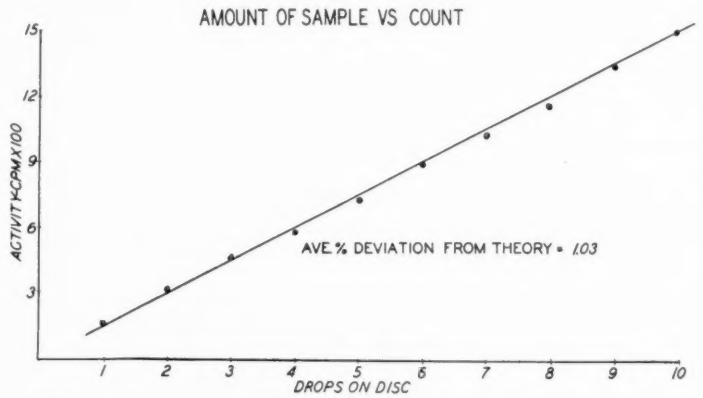

Fig. 3.—Relation of quantity of sample with Na^{22} to count. The line is the theoretically perfect line and the points are the observed values. The rate of counting selected was that usually encountered in the body fluids of man under experimental conditions with "safe" doses of radioactivity.

The influence of the quantity of fluid, within limits, was studied by preparing thirty disks with 1 drop (about 0.023 c.c.) each of a fluid containing Na^{22}, thirty disks with 2 drops each of the fluid, and so forth until a maximum of 10 drops per slide was reached. Results of one such test are summarized by Fig. 3, showing a mean error of 1.03 per cent. Thus the method described is suitable for accurate counting of relatively small volumes of material which vary considerably in concentration of the radioelement. The influence of variations in quantity of material added to the filter paper as well as in concentration of the radioelement was studied for each of the radioelements mentioned. Results were the same as for Na^{22}, summarized by Figs. 2 and 3.

DISCUSSION

The satisfactory reproducibility of preparation of biologic fluids of relatively low radioactivity makes the method useful in radiobiology. The dependability of the preparation of the disks alone is even greater than the values indicate, since the measurements include statistical errors due to counting. A technician can prepare a great number of samples per day accurately after acquiring experience.

With the relatively low level of counting and large numbers of samples that must be studied in some experiments, the experience in this laboratory has been that uniformity of preparation was better and errors due to self-absorption, poor geometric relationship, deliquescence, and other factors were less when filter paper disks were employed than when samples of biologic

fluid were placed on plastic sheets or in metal, glass, or plastic containers. Data comparing these methods have been omitted for purposes of brevity.

This method of preparing samples has been studied sufficiently only for use with glass and mica window counters. It is advisable for observers who undertake this procedure to standardize it for their particular experiments. This is particularly essential if the flow type of counter is used, where absorption of radiation in the preparation is an important varying factor among samples. Since radiomercury ($Hg^{203-205}$) was the element with lowest energy studied, there is no reason to doubt that the method is applicable to radioelements with energies of emission as great or greater than radiomercury. It is advisable, of course, for each investigator to evaluate the procedure for any new radioelement employed.

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A MICROPIPETTE FOR PREPARATION OF SAMPLES FOR COUNTING IN RADIOBIOLOGY

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A PIPETTE has been devised for the delivery of quantities of fluids containing radioelements during the preparation of samples for counting. Since the apparatus is simple and sturdy and since it has proved dependable during four years of constant use, it is described here in the event it may be useful to others.

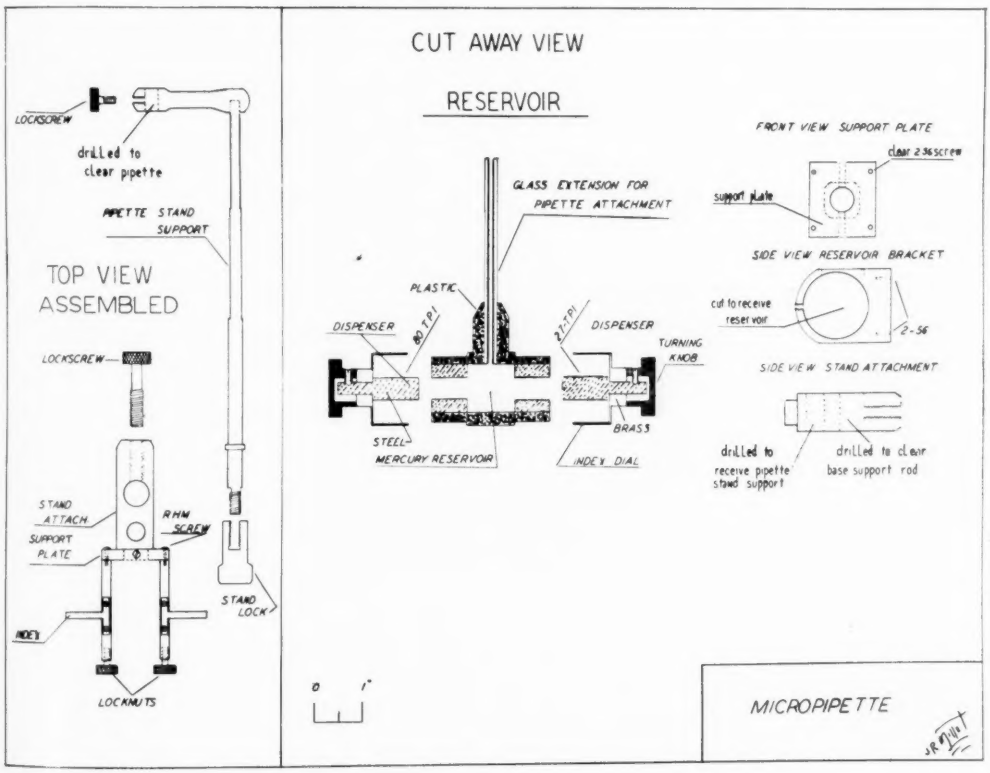

Fig. 1.

Aided by grants from the Life Insurance Medical Research Fund, a War Department Grant No. W-49-007-MD389, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

From the Department of Medicine, Tulane University School of Medicine and Charity Hospital of Louisiana.

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The apparatus is constructed of brass, steel, and Lucite, the pipette itself being of glass. The mercury comes into contact with only glass, Lucite, and steel, difficulties due to amalgamation thus being obviated. The threaded portions of the dispensers are coated with a thin film of stopcock grease to insure a mercury-tight seal. Details of construction are presented in Figs. 1 and 2.

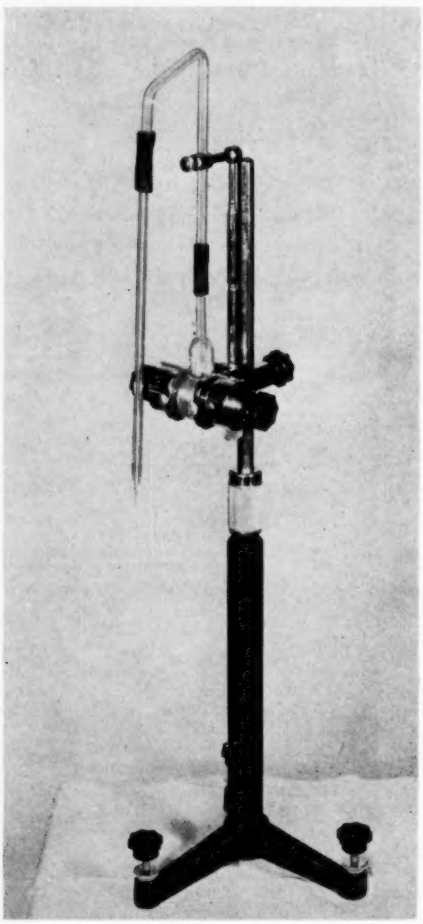

Fig. 2.

By reverse rotation of the dispenser knob the liquid sample is drawn into a 1 c.c. pipette calibrated by the U. S. Bureau of Standards. The size of the pipette may be varied to suit the requirements of the experiments. Either a fine or gross dispenser may be employed, depending upon the volume of fluid to be delivered. When samples are drawn into the pipette, an air column should be maintained between the mercury column from the reservoir and the sample, in order to prevent contamination of the mercury and reservoir. The pipette should be washed between deliveries of samples by expelling the excess material, then successively drawing into the pipette and ejecting from it dis-

tilled water three times from separate beakers, the rinsing water being expelled into a waste beaker. Three rinsings have proved sufficient to clear the pipette of radioelements in all biologic fluids studied in this laboratory. Each observer should verify this for his own type of experiments by drawing a fourth sample of distilled water into the pipette, aliquots of which are counted for contamination.

Because of variations in the physical characteristics of biologic fluids and in room atmosphere, it is advisable to measure the volume of the samples delivered for counting by means of the markings on a calibrated glass pipette. The dispensers are used to control the delivery accurately. It is possible, with proper calibrations and standardizations of technique and fluid to be studied, to employ fine pipettes to deliver known amounts of uniform drops.

The pipette has been standardized by gravimetric and radiometric procedures and it has been found to have a mean error of 0.48 per cent, with a standard deviation of ±0.34 per cent, the maximum error encountered being 2.8 per cent. The interpretation of the significance of these values is difficult when it is realized that they include errors in weighing, counting, and preparation that are not inherent in the pipette itself but in the procedure of calibration. For the usual type of radiobiologic experimental purposes the pipette is, however, considered sufficiently accurate for good work.

IDENTIFICATION OF BLOOD DONORS WITH A FLUORESCENT DYE

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HUMAN blood procurement centers are frequently confronted with the problem of donors who wish to give blood at intervals too frequent for their own physical well-being. Persons of this type are to be found in every group of donors irrespective of the motives which prompt them to come for a blood donation. Experience in all centers where close checks are made have demonstrated that such persons will, in their eagerness, give false statements as to previous donations, give fictitious names, or exchange Social Security cards and other identifying papers. This nullifies the usual recording system. Physical examinations, hemoglobin determinations, and searching for needle marks are also often inadequate for determining whether or not these donors have been bled recently.

A partial solution to this problem has been found in marking donors, at the time of bleeding, with an "invisible" dye. This dye, although truly invisible under ordinary light, fluoresces strongly when exposed to a long-wave, nonerythema-producing ultraviolet illumination.

The dye, CS-20,‡ is a solution of a fluorescent chemical mixture in an isopropanol and water base. The concentration of the dye is on the order of 5 per cent. The mixture is analogous to a dye stain, S-20,‡ which has been widely used, though in somewhat weaker strengths, for marking individuals for pass-out checks in places such as theaters and race tracks. It gives a strong blue fluorescence under ultraviolet rays, the fluorescence being particularly evident when most of the visible light is excluded.

The technique of marking blood donors involves swabbing the dye on a selected finger at the junction of the nail with the cuticle. Capillary attraction causes the dye to penetrate crevices in and under the cuticle where it tends to remain despite repeated washing. It can be readily seen, under a proper lamp, for periods of from two to three weeks and sometimes even longer. It also can be placed in the outer portion of the external ear canal where it remains for a considerable time. The dye appears to be no more irritating to tissues than isopropyl alcohol diluted with water.

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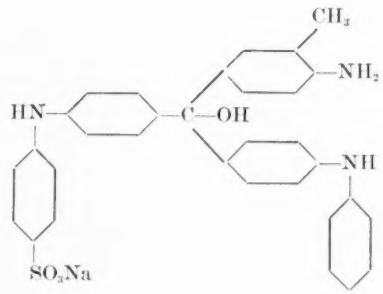
*The Hyland Laboratories.
†Shannon Luminous Materials Company.
‡Dyes CS-20 and S-20 are manufactured by Shannon Luminous Materials Company of Los Angeles.

Although the exact chemical composition of the fluorescent dye, CS-20, is not known, it appears to be closely similar to the colorless carbinol of the dye alkali blue 4B, C.I. 704.



Chemicals of this character and related structure are used extensively for textile dyeing. In this form they have been observed in long-time contact with the skins of countless people without evidence of skin damage.

Several individuals have, to our knowledge, worked with CS-20, S-20, and related dyes for a period of years without making any effort to protect their hands and arms from contact with these stains. There has been no indication among members of this group of skin irritations or other untoward effects due to exposure to these dyes. Stamp pads impregnated with the invisible dye, S-20, have been used to mark a countless number of persons in the past five years at fairs, race tracks, and so on. During this period only one case of alleged irritation has been reported and this proved to be of no significance. Dye S-20 has proved nonirritating as determined by controlled applications to the human skin and observation for intervals extending beyond the period of skin fluorescence when viewed under ultraviolet light.

Dye CS-20, the subject of this report, has not been used as widely as dye S-20. However, dye CS-20 is merely a more concentrated solution of S-20. CS-20 has been applied to the skins of over 75,000 persons during the past ten months, and of these a great number have been seen for reinspection of the treated area. No evidence of irritation has been observed.

Nail-base marking appears to be the most practical since the donor's hand can be examined in a viewing box* so constructed as to cut out extraneous light. The box uses an ultraviolet light, Type EH4, 100 W. high intensity mercury lamp with suitable filters fitted into a circular holder. This in turn fits into a hole in the top of the metal viewing box and can be removed easily by its handle when used for other purposes.

Four blood procurement agencies with bleeding centers in the Los Angeles area are cooperating in a donor-marking program, each laboratory dyeing the nail base of an assigned finger on the left hand at the time of drawing blood. Every prospective donor must have his left hand inspected in a viewing box in addition to the conventional donor requirements before being accepted for bleeding. An observed nail-glow disqualifies the donor. Records are kept and

*Viewing box designed and built by Keese Engineering Company of Los Angeles.

information is exchanged between the cooperating laboratories. In the past ten months over 4,500 prospective donors have been disqualified by this method. Interestingly, it appears that many would-be donors have ceased their attempts to be bled at too frequent intervals since the program of nail-base marking was initiated. A noticeable decrease in the frequency of reactions following blood donation is being observed.

The dye employed is not yet ideal since it cannot always be detected after about three weeks following application. A search is in progress for even more tenacious fluorescent dyes and also for dyes of other colors that will prove satisfactory for skin or nail markings. Dyes other than blue can be identified more easily by ultraviolet light because of the fact that ultraviolet lamps also emit visible blue rays. This report is considered timely, however, in that it describes a method by which four cooperating laboratories have been able to avoid bleeding many would-be donors within intervals often extending up to three weeks and occasionally to as much as six weeks after their most recent donations of blood.

DIRECT IODOMETRIC COLORIMETRIC DETERMINATION OF BLOOD CHLORIDE

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COLORIMETRIC methods based on the iodometric procedure of Sendroy[1] have been described by Sendroy,[2, 3, 4] Hoffman,[5] and Stiff.[6] The method of Stiff simplified the former methods but has not been satisfactory as a routine procedure for the following reasons.—(a) The use of thiosulfate reagent adds an additional step to the method, decreases sensitivity, and prevents the preparation of a single standardization chart. (b) The method is sensitive to small changes in temperature and must be standardized at a controlled temperature or standards run with each set of determinations. The methods of Sendroy and Hoffman also require temperature control.

The method described in this paper does not require the addition of thiosulfate or daily standardization and is insensitive to differences of temperature from 15 to 37°. The method is extremely reproducible.

METHOD

Reagents.—

Phosphoric-Tungstic Acid Reagent. Dissolve 15 Gm. of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) in 1 liter of 0.075N phosphoric acid.

Silver Iodate, C.P. (Powder, chloride free).

0.1N Standard Sodium Chloride Solution. Dissolve 5.845 Gm. of sodium chloride, C.P., which has been dried at 150 to 200° C., and dilute to 1 liter with distilled water.

Sodium Iodide, C.P. (Chloride free).

Standardization.—Dilute 10 ml. of 0.1N sodium chloride to 100 ml. with 50 ml. of phosphoric-tungstic acid reagent and distilled water. In order to simulate the protein-free filtrates as closely as possible, approximately half of the amount of phosphoric-tungstic acid reagent used in the preparation of the protein-free filtrates is used in the preparation of the standards. Add 0, 1, 2, 3, 4, and 5 ml. of this solution to centrifuge tubes and dilute to 5.5 ml. with phosphoric-tungstic acid reagent diluted with an equal portion of distilled water. Add 0.1 Gm. of silver iodate to each tube and continue as directed under Procedure. The spectral transmission curve of the iodine color developed by the chloride standards was linear at 480 mμ with the Coleman spectrophotometers used. Readings may be plotted against concentration of chloride of the standards or a conversion factor (K) calculated for concentration of standard: $C = K \log \frac{1}{T} = -K \log T = K (2 - \log \% T)$.

Procedure.—Add 0.5 ml. of unhemolyzed serum or plasma (or other body fluids) to 5 ml. of phosphoric-tungstic acid in a centrifuge tube. Also add 0.5 ml. of distilled water to 5 ml. of phosphoric-tungstic acid for reagent blank. Add approximately 0.1 Gm. of silver

From the Clinical Biochemistry Laboratory, Wadworth Hospital, Veterans Administration Center.

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iodate with a small spoon spatula to each tube, stopper, and *shake* vigorously for one minute. Centrifuge for five minutes. Add approximately 0.5 Gm. of sodium iodide with a small spoon spatula and dissolve in 20 ml. of distilled water in a colorimeter tube and add 0.5 ml. of clear supernatant. (Standardize approximately the volume of 0.1 Gm. of silver iodate and 0.5 Gm. of sodium iodide in small spoon spatulas.) Let stand for five minutes and read in photometer against reagent blank set at 100 per cent transmission with light of 480 mμ transmission. The color is quite stable and may be read several minutes after color development. Smaller volumes of serum or filtrate may be used with proportionate amounts of reagents as indicated.

Calculation.—Milligrams sodium chloride/100 ml. $= K^1(2 - \log \% T)$, or mM per liter of chloride $= K^2(2 - \log \% T)$.

1. K = 1170 for Coleman spectrophotometers, models 6 and 14, used with No. 6-304B cuvettes.

2. $K = 1170 \times 10/58.46 = 200$.

EXPERIMENTAL

The colorimetric method was compared with the Whitehorn titrimetric chloride method[7] using serum, spinal fluid, urine, and gastric juice (Table I). Good agreement was obtained without any control of temperature or standardization with each set of determinations. Deviations between the two methods ranged from +2.6 to −1.5 mM per liter.

TABLE I. COMPARISON OF THE IODOMETRIC PHOTOMETRIC AND THE WHITEHORN TITRIMETRIC CHLORIDE METHODS (TEMP. 18 TO 26° C.)

COLORIMETRIC METHOD (MM PER L.)	VOLUMETRIC METHOD (MM PER L.)	DIFFERENCE (MM PER L.)	COLORIMETRIC METHOD (MM PER L.)	VOLUMETRIC METHOD (MM PER L.)	DIFFERENCE (MM PER L.)
	Serum		103	102.8	+0.2
105.0	106.2	−1.2	105.0	105.0	0.0
91.0	91.3	−0.3	97.0	98.0	+1.0
96.0	96.8	−0.8	91.0	89.5	+1.5
100.0	100.8	−0.8	101.4	102.7	−1.3
98.0	99.2	−1.2		*Spinal Fluid*	
105.0	104.4	+0.6	113.2	113.5	−0.3
103.0	102.6	+0.4	112.0	113.5	−1.5
93.0	94.0	−1.0	119.0	119.4	−0.4
103.0	104.0	−1.0	117.0	117.4	−0.4
103.0	102.8	+0.2	110.0	110.6	−0.6
100.0	100.8	−0.8	109.0	110.0	−1.0
100.0	100.2	−0.2		*Urine*	
108.0	105.4	+2.6	73.5	74.1	−0.6
96.0	98.0	−2.0	54.7	54.0	+0.7
98.3	99.4	−1.1	75.0	74.2	+0.8
114.6	113.5	+1.1	91.0	91.2	−0.2
104.6	104.2	+0.4	57.0	56.8	+0.2
103.0	102.8	+0.2	77.0	77.0	0.0
104.2	105.0	−0.8	73.0	71.5	+1.5
113.5	113.5	0.0	131.0	132.0	−1.0
88.0	88.0	0.0	62.0	62.0	0.0
103.0	102.8	+0.2	40.2	41.0	−0.8
96.0	97.0	−1.0		*Gastric Juice*	
103.0	103.8	−0.8	40.2	41.0	−0.8
91.0	91.2	−0.2	115.0	113.5	+1.5
105.0	103.1	+1.9			

The effect of temperature was studied by making a complete determination from precipitation of protein to final color development at a fixed temperature. Temperatures of 15°, 25°, and 37° had no appreciable effect on the color development (Table II). No corrections were made for changes in volume.

TABLE II. EFFECT OF TEMPERATURE ON THE IODOMETRIC PHOTOMETRIC METHOD WITH COMPARISON WITH THE WHITEHORN METHOD

SPECIMEN	WHITEHORN (mM PER l.)	PHOTOMETRIC 15° (mM PER l.)	PHOTOMETRIC 25° (mM PER l.)	PHOTOMETRIC 37° (mM PER l.)
Serum	85.4	86.0	86.0	
Serum	93.0	91.0	91.0	
Serum	83.3	81.0	83.0	
Serum	84.0	83.	83.0	
Serum	93.0		93.9	
Serum	93.0		92.6	
Serum	99.6		98.0	
Serum			100.8	100.8
Serum			91.0	91.0
Serum			100.0	100.0
Saline			103.0	100.0
Saline	100.0	99.2	100.0	
Spinal fluid	113.5		112.0	

SUMMARY

A simple, direct, iodometric colorimetric method for the determination of chlorides in body fluids has been described.

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AN IMPROVED METHOD FOR THE DETERMINATION OF AMINO ACIDS IN SMALL SAMPLES OF BLOOD AND URINE

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INTRODUCTION

MICROBIOLOGIC methods for the determination of the specific amino acids have been developed for at least sixteen of the twenty-two amino acids commonly found in protein. These methods have been used to determine the content of specific amino acids of animal and vegetable proteins, such as plasma protein fractions, hemoglobin, and muscle proteins.[1] These methods have also been adapted for the determination of the content of the specific amino acids in the nonprotein fraction of plasma, sweat, cerebrospinal fluid, and urine.[2-7] The relatively large volumes of blood (40 to 50 ml.), cerebrospinal fluid (40 to 80 ml.), sweat (10 ml.), and urine (30 to 50 ml.) necessary for the determination of eleven of the individual amino acids in the same sample limits the value of microbiologic methods in the study of amino acid metabolism in small laboratory animals or clinical studies in infants and children.

A technical modification of the microbiologic assay principle, which permits the determination of at least eleven of the specific amino acids using only 12 to 15 ml. of whole blood, is presented. This method, in addition to the small size of sample necessary, utilizes an economical and a rapid method of media preparation, a rapid "drop method" for accurately measuring standard and unknown samples, and a time-saving method of preparing the samples for incubation. As a result, this method has proved to be useful for clinical investigation over a period of four years.

METHOD

1. *Preparation of the Synthetic Media.*§—The synthetic medium used is a modification of that recommended by Sauberlich and Baumann.[8] A different medium is required for each of the amino acids to be determined. To facilitate the preparation of the synthetic media, the amino acid fractions, the glucose-buffer fractions, and the salt-vitamin fraction are prepared in advance.

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§Complete, dry media, requiring only the addition of water, are available from the H. M. Chemical Co., Ltd., Los Angeles, Calif. Preliminary studies reveal some of these to be adequate for use in amino acid assays of plasma filtrates.

(a) The Amino Acid Fractions: The amino acid fraction for each amino acid is prepared by using the quantities of amino acids listed in Table I. The specific amino acid to be determined is omitted. After weighing the amino acids they are mixed and powdered thoroughly in a mortar. Each fraction is then stored at room temperature in the dry state in a tightly stoppered bottle. When prepared according to the amounts listed in Table I, each amino acid fraction is sufficient for the preparation of approximately five thousand assay tubes.

(b) Glucose-Buffer Fractions: Since the various organisms require different buffering agents for optimum growth, two glucose-buffer solutions are used: a glucose-acetate solution and a glucose-citrate solution, Table I. These solutions are prepared fresh for each assay but are satisfactory for as long as four weeks if properly stored at 4° C.

(c) Salt-Vitamin Fraction: Solutions of salts and vitamins are prepared separately and combined after complete solution in their respective solvents. The salts, purines, and pyrimidines for the media are listed in Table I. This mixture is dissolved in 100 ml. of 1 normal hydrochloric acid. The vitamins required are listed in Table I. Following weighing, the vitamin mixture is dissolved in 100 ml. of distilled water. The two solutions are then mixed and placed in amber glass bottles. This is the salt-vitamin fraction. It is stored in the frozen state until needed. It is essential that the mixture of salts, purines, and pyrimidines be dissolved in hydrochloric acid before mixing with the vitamin solution. Complete solution of all components is difficult unless this procedure is followed.

TABLE I. MEDIA FOR MICROBIOLOGIC ASSAY OF AMINO ACIDS

1. AMINO ACID FRACTION

AMINO ACID*	FRACTION I	FRACTION II	FRACTION III
DL-leucine	1,000 mg.	1,000 mg.	1,000 mg.
DL-isoleucine	1,000 mg.	1,000 mg.	1,000 mg.
DL-valine	1,000 mg.	1,000 mg.	1,000 mg.
L-cystine	500 mg.	1,000 mg.	1,000 mg.
DL-methionine	500 mg.	500 mg.	1,000 mg.
L-tryptophane	250 mg.	500 mg.	500 mg.
L-tyrosine	250 mg.	500 mg.	500 mg.
DL-phenylalanine	500 mg.	500 mg.	500 mg.
DL-glutamic acid	2,000 mg.	2,000 mg.	2,000 mg.
DL-threonine	1,000 mg.	1,000 mg.	1,000 mg.
DL-alpha-alanine	1,000 mg.	500 mg.	1,000 mg.
DL-asparagine	1,000 mg.	1,000 mg.	1,000 mg.
L-lysine·HCl·H_2O	1,000 mg.	1,000 mg.	1,000 mg.
L-arginine·HCl	250 mg.	250 mg.	500 mg.
L-histidine·HCl·H_2O	250 mg.	250 mg.	500 mg.
DL-serine	250 mg.	250 mg.	1,000 mg.
Glycine	100 mg.	100 mg.	500 mg.
L-proline	250 mg.	250 mg.	250 mg.

2. GLUCOSE-BUFFER FRACTION

GLUCOSE-ACETATE (SOLUTION A)		GLUCOSE-CITRATE (SOLUTION B)	
Glucose	50 Gm.	Glucose	50 Gm.
Sodium acetate	50 Gm.	Sodium citrate·H_2O	67.5 Gm.
Distilled water to	250 ml.	Distilled water to	250 ml.

3. SALT-VITAMIN FRACTION

A. SALTS, PURINES, AND PYRIMIDINES		B. VITAMINS	
KH_2PO_4	2,000 mg.	Thiamine·HCl	2.0 mg.
K_2HPO_4	2,000 mg.	Pyridioxine·HCl	4.0 mg.
$MgSO_4·7H_2O$	800 mg.	Ca pantothenate	2.0 mg.
$FeSO_4·7H_2O$	40 mg.	Riboflavin	2.0 mg.
$MnSO_4·4H_2O$	40 mg.	Nicotinic acid	4.0 mg.
NaCl	40 mg.	p-Aminobenzoic acid	0.4 mg.
Adenine $SO_4·2H_2O$	40 mg.	Biotin	0.004 mg.
Guanine HCl·$2H_2O$	40 mg.	Folic acid	0.04 mg.
Uracil	40 mg.	Distilled water	100 ml.
Xanthine	40 mg.		
1N hydrochloric acid	100 ml.		

*The amino acid to be assayed is omitted.

The synthetic media are prepared by combining measured portions of the three fractions and distilled water. The quantity of each necessary is proportional to the number of tubes required for the assay. Each one hundred amino acid determinations (one hundred tubes) requires approximately 250 mg. of the appropriate amino acid fraction. This is dissolved in 5 ml. of 1 normal hydrochloric acid. To this, 10 ml. of the appropriate glucose-buffer fraction, 5 ml. of the salt-vitamin solution, and 5 ml. of distilled water are added. This provides a total volume of 25 milliliters. The final solution is adjusted to pH 6.8 by adding sodium hydroxide, using bromthymol blue as an external indicator.

2. *Preparation of Standard Amino Acid Solutions.*—Approximately 40 mg. of the specific amino acid to be determined are weighed accurately and dissolved in a sufficient volume of 1 normal hydrochloric acid to provide a concentration of 1 mg. of amino acid per milliliter. An aliquot of this solution is then diluted appropriately, Table II. The final solution is then adjusted to pH 6.8 with sodium hydroxide solution, using bromthymol blue as the indicator. This solution is divided into 20 to 25 ml. portions and placed in 25 ml. penicillin bottles. This standard solution can be stored for an indefinite period in the frozen state at low temperatures (approximately minus 40° C.). There is no significant change in the concentration of the solutions even after six months of storage.

3. *Collection and Preparation of the Plasma Filtrate.*—The blood specimen is placed in a concentrated solution of mixed oxalate to prevent clotting (1 drop for each 5 ml. of whole blood)* and centrifuged immediately, as standing or clotting may influence the amino acid content of the plasma.[2] The plasma is then separated and weighed. The volume is calculated by dividing the weight of the plasma by 1.026, the assumed specific gravity of plasma. This is done to obtain the maximum amount of plasma for analytical purposes.

The classical tungstic acid procedure for the preparation of a protein-free filtrate is modified slightly. The plasma filtrate is prepared by adding to 1 volume of the plasma 3 volumes of 0.07 normal hydrochloric acid and 1 volume of a 2.5 per cent sodium tungstate solution. The plasma is thoroughly mixed during the addition of the hydrochloric acid and the sodium tungstate. The mixture is filtered through Whatman No. 40 filter paper. The filtrate is then neutralized with 1 normal sodium hydroxide using bromthymol blue. This provides a filtrate with the lowest biuret color obtainable for this concentration of tungstate, as measured in a spectrophotometer. All the amino acids are determined without further dilution. The tungstic acid filtrate prepared as described produces no inhibition or enhancement of the growth of the organisms used. If it is desired to determine the amino acids at a later time, the filtrate is stored in the frozen state. A convenient method is to divide the filtrate into several portions and to place each portion in chemically clean 25 ml. penicillin bottles, cap with the fitted rubber stopper, and freeze immediately. The use of fractional parts of the samples permits assay at a later date of any number of the amino acids without danger of contaminating the remainder of the sample.

4. *Collection and Preparation of Urine Samples.*—Urine samples are collected in bottles containing a mixture of 2 ml. of toluene and 10 ml. of 1 normal hydrochloric acid. The bottle should be kept in ice water during collection. Highly concentrated urines should be adjusted to approximately 1,500 ml. volumes. A large volume of dilute urine need not be adjusted. Highly concentrated urines may inhibit growth of organisms unless properly diluted.

The "free" amino acid content of the urine is determined on dilutions of 1:1 or 1:5. The dilution used is determined on the basis of the expected "free" amino acid content of the urine sample, Table II. The urine is adjusted to pH 6.8 with sodium hydroxide and filtered before the final solution is made. The total amount of the specific amino acid ("free" plus "conjugated") in the urine sample is determined on hydrolyzed urine. Acid hydrolysis is used for all amino acids except tryptophane which requires alkaline hydrolysis. The urine for acid hydrolysis is prepared by the addition of 3.2 ml. of a 1:1 solution of concentrated sulfuric acid to a 25 ml. aliquot of the urine sample. For alkaline hydrolysis

*The mixed oxalate recommended by Heller and Paul[9] is used. The concentrated solution is prepared by dissolving 3.6 Gm. of ammonium oxalate and 2.4 Gm. of potassium oxalate in 35 ml. of distilled water.

TABLE II. ORGANISM, AMINO ACID FRACTION, GLUCOSE-BUFFER, CONCENTRATION OF STANDARD SOLUTION, CONDITIONS FOR AUTOCLAVING, DROPS SAMPLE PER TUBE, AND THE DILUTION OF URINE FOUND TO BE MOST SATISFACTORY FOR ASSAY

AMINO ACID	ORGANISM	AMINO ACID FRACTION	GLUCOSE-BUFFER FRACTION	CONC. OF STANDARD SOLUTION* (μG PER ML.)	CONDITIONS FOR AUTOCLAVING		PERIOD OF INCUBATION (HR.)	VOLUME OF PLASMA FILTRATE PER TUBE (DROPS)	URINE DILUTION		VOLUME OF URINE PER TUBE (DROPS)
					MIN.	LB. PRESS.			FREE	TOTAL	
Arginine·HCl	Str. faecalis	I	A	5 or 10	10	15	36-50	2 to 12	1:5	1:10	2 to 14
Histidine·HCl·H_2O	Str. faecalis	I	A	10 or 20	10	15	36-50	3 to 9	1:5	1:50	2 to 14
Isoleucine†	L. arabinosus	I	A	10 or 20	10	15	36-50	7 to 15	1:1	1:5	2 to 14
Leucine	L. arabinosus	I	A	10 or 20	10	15	36-50	7 to 15	1:1	1:5	2 to 14
Lysine·HCl·H_2O	L. mesenteroides	III	A	5 or 10	5	15	36-50	5 to 11			
	Str. faecalis	II	B	5 or 10	5	15	50-72	5 to 11			
Methionine	Str. faecalis	II	B	5 or 10	10	15	50-72	4 to 10	1:5	1:5	2 to 14
Phenylalanine	L. arabinosus	I	A	5 or 10	10	15	36-50	3 to 15	1:5	1:10	2 to 14
Threonine	Str. faecalis	II	B	5 or 10	5	10	36-50	3 to 7	1:5	1:5	2 to 14
Tryptophane	L. arabinosus	I	A	1 or 2	10	15	36-50	1 to 5	1:5	1:5	2 to 14
Tyrosine	L. arabinosus	I	A	5 or 10	10	15	36-50	3 to 15	1:5	1:10	2 to 14
Valine	L. arabinosus	I	A	10 or 20	10	15	50-72	7 to 15	1:1	1:10	2 to 14

*Expressed in micrograms per milliliter of biologically active amino acid.
†4.0 μg of DL-isoleucine were added to each milliliter of synthetic media.

1 ml. of 12 normal sodium hydroxide is added to a 5 ml. aliquot of the urine. The solutions are placed in 125 ml. Erlenmeyer flasks, covered with a 50 ml. beaker, and autoclaved for five hours at 15 pounds pressure. The solutions are then cooled and neutralized to pH 6.8 with 15 normal sodium hydroxide and 12 normal hydrochloric acid respectively, using bromthymol blue as an external indicator. The hydrolyzed urine samples are diluted with sufficient distilled water to provide a 1:2 dilution of the original sample. The hydrolyzed urine samples are then filtered and stored in the frozen state. Further dilutions are made at the time of assay, depending on the specific amino acid to be determined (Table II).

5. *Bacteria.—Lactobacillus arabinosus* (17-5),* *Streptococcus faecalis* R,* and *Leuconostoc mesenteroides* (P-17)* are used as the assay organisms. Stab cultures of the organism are maintained on enriched media and stored at 4° C.† The stock cultures are transferred once each month to new media by first transferring to enriched broth media and incubating for twenty-four hours at 37.5° C. The new stab cultures are made from the broth culture by transferring to an enriched agar slant and incubating for twenty-four hours at 37.5° C. The slants are then sealed with tight-fitting rubber stoppers and stored at 4° C.

6. *Procedure.*—Assays are prepared by filling test tube blocks‡ with meticulously cleaned 13 mm. by 100 mm. Pyrex rimless bacteriologic test tubes. Each tube is labeled with a wax pencil. The unknown filtrate samples and the standard amino acid solutions are added to the test tubes using the same 2 ml. Mohr pipette. The tubes for the preparation of the standard curve for the amino acid to be determined are prepared first.

The standard amino acid solution for the specific amino acid to be determined is added to the tubes in the following manner. To the first two tubes no standard solution is added; then, 1 drop is added to each of the next two tubes and 2 drops to each of the next two tubes and so on, increasing by 1 drop for each subsequent pair of test tubes until 14 drops have been added to each of the last two tubes.

Three to eight tubes are required for each unknown sample to be determined. Using the same Mohr pipette as for the standard solution, a varying number of drops of the unknown filtrate such as 1, 3, 5, 7, and 9 or 2, 5, 8, 11, and 14, and so on, are added to each of the tubes. Plasma values for individual amino acids do not vary widely from sample to sample, therefore, only three to five tubes are necessary, while urine filtrates may require as many as eight tubes. Following the filling of each series of tubes, the pipette is drained on gauze and refilled with the succeeding sample. Since all samples are closely similar in amino acid content, the error due to the residuum of the previous sample in the pipette is considered negligible. The volume of each tube is adjusted to a total of 15 drops by the addition of distilled water from a 10 ml. pipette. Thirty to thirty-two of these tubes are then placed in No. 2 vegetable cans (8½ cm. by 11½ cm.). The cans are covered with a 10 cm. Petri dish cover to prevent contamination with dust particles. Approximately 20 ml. of water are added to the bottom of each can to facilitate heat conduction and to reduce evaporation from the assay tubes. It is important to place the tubes for each amino acid to be determined in separate cans and to keep them covered and cool until the appropriate amino acid-deficient medium is added. After all tubes have been placed in the tin cans, 5 drops of the appropriate amino acid-deficient synthetic medium are then added to each of the tubes without removing the tubes from the cans. A 10 ml. pipette is used to deliver the drops of amino acid-deficient medium.

The assay tubes are then autoclaved in the cans for a suitable period (Table II) and then cooled to room temperature. A twice washed saline suspension of a twenty-four hour broth culture of the assay organism is then prepared and diluted to three times the original broth culture volume. Under aseptic conditions, 1 drop of the suspension is added to each

*Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

†Enriched broth culture medium is prepared by adding 1.0 Gm. glucose, 0.5 Gm. Bactopeptone, 0.6 Gm. sodium acetate, 0.3 Gm. beef extract, 2.5 ml. of the vitamin-salt fraction, and 20 mg. of cystine (initially dissolved in HCl) to 100 ml. of distilled water. The solution is neutralized to pH 6.8 with sodium hydroxide. The enriched agar medium is prepared by dissolving 1.5 Gm. of agar in 100 ml. of the enriched broth.

‡One time-saving advantage is the use of hard maple blocks as test tube holders. These contain sixty holes, 15 mm. in diameter and 30 mm. deep. The blocks have two sets of holes of three rows each, ten holes to a row. This permits separation of each group of amino acid determinations to be done on the unknown filtrate.

tube. The Petri cover is replaced and the tubes are incubated in the cans at 37.5° C. The period of incubation depends on the organism used and the amino acid being determined, Table II. Following incubation, the cans are subjected to live steam at atmospheric pressure for twenty minutes and then cooled to room temperature. The individual tubes are titrated with carbon dioxide-free 0.02 normal sodium hydroxide to determine the amount of acid produced. Bromthymol blue, 0.25 per cent, is used as an internal indicator.

The synthetic media, buffer, the assay organism, concentration of the standard solution, number of drops of filtrate of plasma and urine, and the conditions for autoclaving for the individual amino acids are given in Table II.

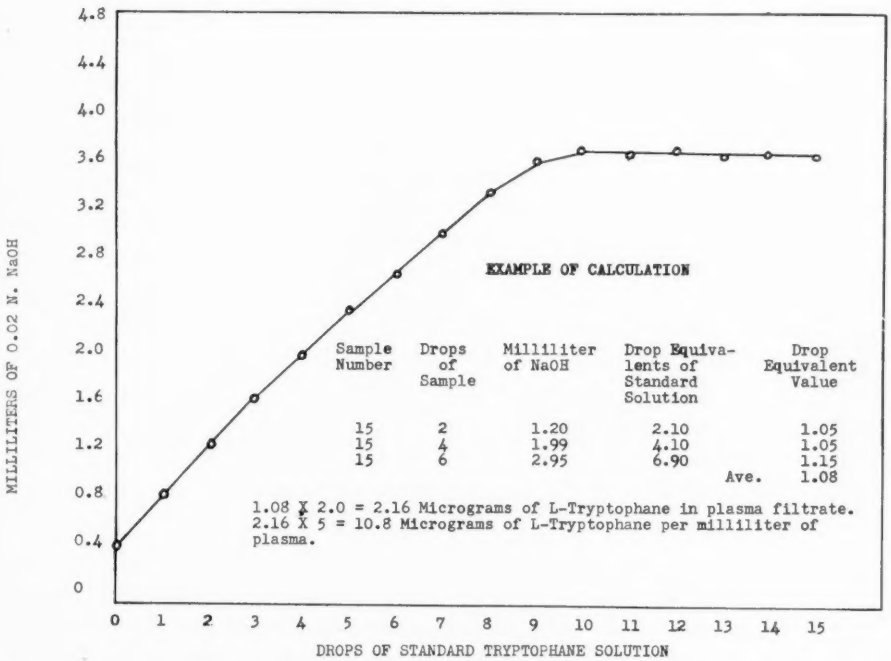
MILLILITERS OF 0.02 N. NaOH
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DROPS OF STANDARD TRYPTOPHANE SOLUTION
EXAMPLE OF CALCULATION
Sample Number | Drops of Sample | Milliliter of NaOH | Drop Equivalents of Standard Solution | Drop Equivalent Value
15 | 2 | 1.20 | 2.10 | 1.05
15 | 4 | 1.99 | 4.10 | 1.05
15 | 6 | 2.95 | 6.90 | 1.15
Ave. 1.08
1.08 X 2.0 = 2.16 Micrograms of L-Tryptophane in plasma filtrate.
2.16 X 5 = 10.8 Micrograms of L-Tryptophane per milliliter of plasma.

Fig. 1.—Standard tryptophane curve with an example of the method of calculation. The concentration of the standard L-tryptophane solution is 2 μg per milliliter.

7. *Calculations.*—A standard curve for each amino acid is made by plotting on the ordinate the number of milliliters of 0.02 normal sodium hydroxide required to neutralize the acid formed in each tube against the number of drops of standard solution that was added to the tube, plotted as the abscissa (Fig. 1). The titration value of each unknown sample is used to determine the amount of amino acid present. The titration value in milliliters of 0.02 normal sodium hydroxide for each unknown sample is found on the ordinate of the standard curve. The corresponding number of drops of standard solution required to produce a similar amount of acid is then located on the abscissa of the standard curve. The number of drops thus obtained, as the titration equivalent, is divided by the number of drops of filtrate added to the unknown. This is the equivalent of each drop of unknown expressed in drops of standard solution. The concentration of standard solution is known, therefore the amino acid concentration of the unknown sample can be determined by the following equation:

$$\frac{\text{Equivalents of each unknown in drops of standard solution}}{\text{Drops of each unknown solution}} \times \text{Concentration of standard amino acid expressed in micrograms per milliliter} \times \text{(Dilution factor)} = \text{Micrograms per milliliter in the unknown sample}$$

ACCURACY OF THE DROP METHOD

The accuracy of the drop method was tested in the following manner. Assays on duplicate plasma filtrates prepared separately from the blood of four young healthy male subjects in a fasting state were made. Ten amino acids were determined. The mean per cent variation for each of these duplicate assays is presented in Table III. The highest mean per cent variation for the ten amino acids determined was 5.5 per cent.

TABLE III. PER CENT VARIATION OF DUPLICATE FILTRATES PREPARED FROM SAME PLASMA; OBSERVATIONS ON BLOOD SAMPLES FROM FOUR SUBJECTS

AMINO ACID	SUBJECT				MEAN VARIATION (PER CENT)
	1	2	3	4	
Arginine	1.2	9.6	1.8	5.4	4.5
Histidine	3.4	0.6	12.1	4.2	5.1
Isoleucine	8.1	0.0	2.2	6.8	4.3
Methionine	9.2	3.4	3.4	6.2	5.5
Phenylalanine	8.2	0.2	1.0	3.2	3.2
Threonine	1.2	0.8	4.2	10.2	4.1
Tryptophane	7.5	3.0	3.2	3.5	4.3
Tyrosine	2.2	6.6	4.1	8.0	5.2
Valine	0.8	0.2	11.5	9.2	5.4

Further test of the accuracy of the method was made by recovery experiments. A solution of eleven amino acids of sufficient concentration to about double the original plasma level for each amino acid was added to plasma. Filtrates were then prepared and the amino acid content of the original plasma and the plasma containing the added amino acids was determined. The per cent recovery values are presented in Table IV. It will be noted that the standard deviation of the mean per cent variation for four determinations ranged between ±4.5 and ±12 for nine amino acids determined.

A comparison of the values obtained in normal adults by Hier and Bergeim[7] with the values obtained by the drop method for normal children and growing albino rats (Sprague-Dawley) is presented in Table V. Observations have been made on twenty-one normal children from 3 months to 15 years of age. Age differences of a significant nature are not observed for

TABLE IV. PER CENT RECOVERY OF AMINO ACIDS ADDED TO PLASMA

AMINO ACID	PLASMA SAMPLE				MEAN	STANDARD DEVIATION
	1	2	3	4		
Arginine	115	82	86	91	94	9.5
Histidine	109	94	89	108	100	8.5
Isoleucine	120	112	122	92	112	9.5
Leucine	121	102	93	98	104	9.0
Methionine	89	78	92	85	86	4.5
Phenylalanine	86	93	110	97	94	7.0
Threonine	92	72	101	88	88	8.5
Tryptophane	113	92	115	96	104	10.0
Tyrosine	128	104	88	112	108	12.0

arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane, tyrosine, and valine. Therefore the values for normal children presented in Table V include all ages.

TABLE V. FASTING PLASMA VALUES OF INDIVIDUAL AMINO ACIDS (REPORTED AS MICROGRAMS PER MILLILITER)

AMINO ACID	NORMAL CHILDREN*		NORMAL ADULTS (HIER AND BERGEIM[7])		ALBINO RATS†	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Arginine	23.5	5.5	23.4	6.2	32.2	3.1
Histidine	18.2	3.4	14.2	1.8	18.8	2.3
Isoleucine	16.3	2.5	16.0	3.2	21.2	2.1
Leucine	18.9	2.5	19.1	3.4	28.9	3.1
Lysine			29.5	4.2		
Methionine	9.9	2.0			10.8	1.1
Phenylalanine	9.6	1.6	13.8	3.2	12.7	1.0
Threonine	14.9	1.5	20.2	4.5		
Tryptophane	10.6	1.2	10.8	2.1	17.3	1.2
Tyrosine	9.9	1.8	14.8	3.7	15.4	1.2
Valine	25.2	4.4	28.3	3.4	28.9	3.8

*Each value represents the average of ten to twenty children.

†Each value represents the average of six aliquots of three to six male rats in the aliquots.

SUMMARY

1. A technical modification of the microbiologic assay method for determining individual amino acids in small volumes of urine and plasma has been presented.

2. A timesaving method of preparation of the samples for assay and the use of a rapid drop method for measurement of volumes are described.

3. Recovery experiments and reproducibility of results in replicate assays for nine amino acids are reported.

4. The values of ten "free" amino acids in the plasma of apparently normal children and for nine "free" amino acids in normal Sprague-Dawley rats are compared with those reported in the literature.

5. These modifications permit more general use of the microbiologic amino acid methods in the average laboratory.

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NONAUTOMATIC PIPETTING DEVICES FOR THE MICROBIOLOGIC LABORATORY

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LABORATORY infections are rather common among bacteriologists working with pathogenic microorganisms. An unpublished compilation at this laboratory lists 875 infections which are recorded in the literature and 66 more unrecorded. Among the viral and rickettsial diseases, 222 laboratory infections have been summarized recently.[1] Everyone who has been long in the field of medical or veterinary microbiology knows from his own experience of a few unpublished cases.

Often the accident responsible for the illness is known, but not always so. Indeed, experience at Camp Detrick is that most such occupational illnesses are not traceable to definite breaks in technique. It is now known that many common bacteriologic operations create hazardous aerosols which may be responsible.[2, 3] Aspiration of infectious fluids through a pipette is one of the well-recognized, obviously dangerous mishaps.

There are at least two steps which may be taken to reduce the danger of sucking injurious fluids into the mouth. The first is the common practice of plugging the proximal end of the pipette with absorbent cotton. The second is not common but is completely effective, namely, avoiding direct oral suction on the pipette.

The use of a rubber bulb was made mandatory during the war at the laboratories of the United States Navy Medical Research Unit No. 1, University of California, Berkeley. There ". . . personnel eventually became very efficient in manipulation and . . . accuracy with this technique was of an order suitable for all bacteriological work. Certainly the pipetting error with 1 ml. pipettes did not exceed 10 per cent."[4] This bulb is probably the cheapest and most easily available device.

Another method involves the use of a rubber tube. One end is attached to the pipette and the other, attached to a mouthpiece made of glass tubing, is put in the mouth. A glass bulb with cotton may be interposed in the tubing as an additional safety measure. This procedure has been common in several laboratories and was first called to our attention by Dr. E. H. Lennette. "With a little practice one can do fairly well, although I must admit that with small volumes the speed of flow, and therefore the accuracy, may be difficult to control."[4]

Another arrangement, which is in use in some laboratories handling radioactive material, utilizes a vacuum line connected to a hypodermic needle pushed through a rubber stopper.* The rubber stopper containing the needle is pulled

From the Biological Department, Chemical Corps, Camp Detrick.
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*Developed at the Oak Ridge National Laboratory by Mr. Leon Wish.

down to apply vacuum to the pipette. If the needle is adjusted to protrude through the stopper about one-half inch into the pipette, there is no danger that an active solution will reach the top of the pipette where it can come in contact with the finger of the operator. When fluid reaches a suitable level, the stopper is released. It is quickly pulled out of the way by the effect of spring tension connected with a pantograph type of holder. The index finger of the operator is immediately placed over the opening of the pipette and fluid is controlled in the usual fashion.

Fig. 1.

Two pipettors, the Caulfield* and the Fisher,† have been commercially available for some time. In our experience neither will conveniently deliver to 0.01 milliliter. They are useful for some purposes, however, and are not expensive. There may be other nonautomatic pipetting instruments suitable for 0.1 ml. to 10 ml. infectious fluids but they have not come to our attention.

The Sterling Hand Pipette‡ and a few others of this type may be good for routine operations with noninfectious material but are not adapted to miscellaneous, varied, intermittent or occasional pipettings of different highly infectious fluids.

Because of the limited acceptability of the devices already mentioned, a systematic attempt has been made to develop other items. The first of these which has been completed is a modification of the Rosen Micropet, Fig. 1.§ This is made of anodyzed aluminum and is easily disassembled. It will deliver accurately 0.01 ml. in a 1 ml. pipette or 0.1 ml. in a 10 ml. pipette and can be operated with one hand while the third and fourth fingers hold a cotton plug from a test tube. Persons who have poor control of the thumb and forefinger may need to use both hands. To fill a 10 ml. pipette, the bulb must be squeezed

*Caulfield Safety Devices, 1512 Packard Bldg., Philadelphia, Pa.
†Fisher Scientific Co., 711 Forbes St., Pittsburgh, Pa.
‡Ivan Sorvall, Inc., 210 Fifth Avenue, New York 10, N. Y.
§S. Rosen and Associates, 5005 Queensbury Ave., Baltimore 15, Md.

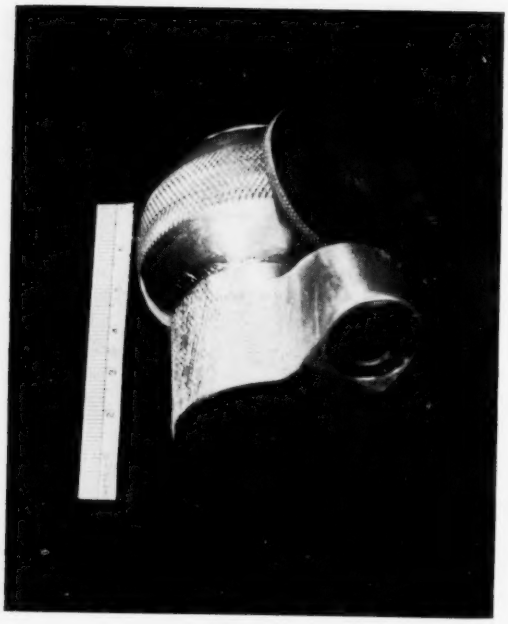

Fig. 2.

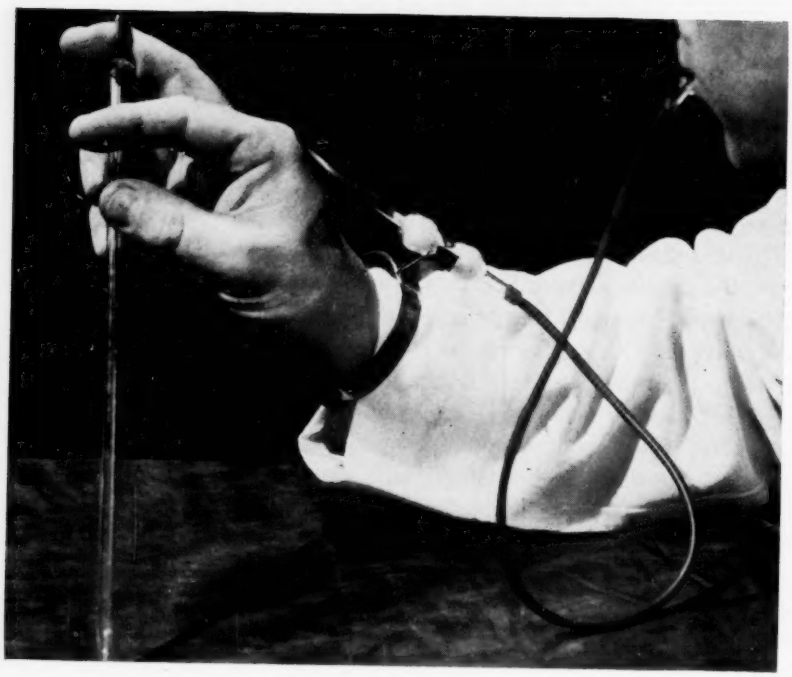

Fig. 3.

twice. Some persons prefer to remove the rubber bulb and attach a vacuum line. Repeated autoclaving probably will lessen the sensitivity of the inner valve core so gaseous disinfection is preferable if the inner mechanism is contaminated. However, the cores are inexpensive and replaceable.*

A second device† is illustrated in Fig. 2. This is not commercially available, but can be built by a competent machinist. Labor cost would be high. It is essentially a rack and pinion operating a piston. The receptor for the pipette is the same as on the Rosen Micropet. This co-called "Rawson" pipettor also requires only one hand, is as accurate in delivery as the Rosen, will fill a 1 ml. or 10 ml. pipette in one turn of the wheel, and the flow of liquid is more easily controlled. Two fingers can be freed to hold cotton plugs. It can be autoclaved without injury but would then have to be taken apart and regreased. Its principal disadvantages are lack of commercial availability, high cost, and increased weight. A modified design is now being made which employs the same principle of operation but is suitable only for pipettes with a capacity of 1 ml. or less.

A third pipetting device is the R-M finger clip,† Fig. 3. This likewise is not commercially available but is made easily at a low cost in material and time. It has no moving parts, does not tire the thumb as will either a bulb or wheel when there are many pipettings to do, and is not injured by autoclaving. The pipette is held against the receptor and is not inserted as in the others described. The principal disadvantage is that it is not entirely safe. When highly infectious or toxic materials are present, the mouthpiece may unknowingly become accidentally contaminated. It is, however, very simple to use and requires no retraining in manual control. A modification which has been found particularly useful in a closed bacteriologic transfer cabinet is one in which the small rubber tubing is replaced by a vacuum line carrying a slight continuous suction and the device is clamped firmly to an independent rigid support. In the arrangement the pipette is held against the receptor until the height of the fluid is satisfactory, then the pipette is quickly removed and controlled by the finger tip as usual; this method eliminates oral suction.

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*The valve core which is used is #100A, made by the Dill Mfg. Co., 700 East 82nd St., Cleveland 8, Ohio. It may be necessary to try four or five cores before one is found which is sufficiently sensitive to deliver 0.01 ml. conveniently. It is better to obtain a tested replacement from S. Rosen.

†Designed at Biological Laboratories, Camp Detrick.

THE USE OF A SEMISOLID MEDIUM FOR DETECTION OF MOTILITY AMONG THE ANAEROBES

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THE general procedure for determining motility of members of the genus Clostridium is the hanging drop method.[1] This method utilizes a hanging drop slide, cover slip, and a young anaerobic culture of from 12 to 18 hours old. Motility is determined by observing a drop of the culture under the high power of the microscope. If motility is doubtful or apparently negative, a still younger culture 4 to 6 hours old may be used. Several attempts usually must be made before motility can be definitely established.

MATERIAL AND METHOD

A modified semisolid agar[2] for determining motile cultures of Salmonella and for obtaining extremely motile cultures for preparation of "H" serums was used with excellent results in motility experiments with cultures of Clostridium. The semisolid medium was dispensed in 8 ml. amounts in a small-bore tube, 14 to 15 mm. by 145 to 150 mm.; just before inoculation the tube of medium was boiled for about ten minutes, and while still hot 0.15 ml. of a sterile 20 per cent solution of sodium thioglycollate was added. When cool, a stab of about 12 to 15 mm. deep was made using a small looped needle. This provided sufficient inoculum to assure growth in the medium. The age of the culture used as inoculum apparently was not a factor since stock cultures of from 24 hours to 6 months old were used without any noticeable difference in the motility determination.

After twenty-four to forty-eight hours of incubation at 37° C., motile organisms spread toward the bottom of the tube, and the degree of motility was estimated by the depth to which growth was apparent during a given period of incubation. The nonmotile organisms grew only along the line of stab, while the remainder of the medium was clear. Organisms of doubtful motility produced offshoots from the growth in the line of stab. Upon prolonged incubation a spreading haze appeared in the environment of the stab, indicating slight motility.

RESULTS

Nineteen members of the genus Clostridium were tried using the semisolid medium. Spreading growth was heavy after twenty-four hours of incubation with cultures of *Cl. tetani, Cl. botulinum, Cl. histolyticum, Cl. oedematoides, Cl. oedematiens,* and *Cl. sporogenes.* Light spreading growth was obtained with cultures of *Cl. sphenoides, Cl. tertium, Cl. septicum,* and *Cl. tetanomorphum.* Cultures of *Cl. perfringens* (*welchii*) produced only heavy growth along the line of stab.

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An advantage of this method of motility determination lies in the development of active motility in less motile strains of Clostridium. Weakly motile organisms require several days of incubation before growth spreads to the bottom of the tube. The upper part of the medium may be heated and allowed to run out of the tube leaving a small amount of an actively motile culture in the base, and serial transfers can be made until growth spreads to the bottom of the tube of the semisolid medium in twenty-four hours.

Cultures which yield extremely motile organisms with a maximum development of "H" or flagellar antigen have been obtained by using the foregoing procedure. This method is now being used in an agglutination study of Clostridium cultures.

Even though this semisolid medium contains a considerable quantity of gelatin, no liquefaction by the anaerobes has been observed.

SUMMARY

A simple method of determining motility of anaerobic bacteria is described. Spreading growth apparent in a semisolid medium constitutes a positive test, while growth only along the line of stab is a negative test. The semisolid medium also can be used to produce extremely motile organisms.

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USE OF TETRATHIONATE BROTH IN ISOLATION OF SALMONELLA, SHIGELLA, AND PARACOLON BACILLI

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TETRATHIONATE broth has proved valuable in the examination of fecal specimens wherever tried. The present data adds one more favorable report to the record and submits information concerning the use of tetrathionate broth in the isolation of a paracolon of probable pathogenicity and other possibly significant organisms.

The paper of Galton and Quan,[6] who first used tetrathionate broth in a public health laboratory in this country, summarizes reports from other countries. Mueller[9] in 1923 was the first to employ this medium, showing that it inhibited coli and allowed typhoid and paratyphoid organisms to grow. Kauffmann[8] modified it by the addition of bile and brilliant green and in 1935 reported the results of 10,182 fecal examinations employing the new medium. He found an increase of Salmonella isolations of 700 per cent over previous findings and an increase of 30 to 40 per cent in isolation of the typhoid bacillus, while isolation of dysentery bacilli increased 10 to 20 per cent. However, he recommended streaking the broth after five hours of incubation in order to isolate dysentery bacilli.

Hormaeche and Peluffo[7] gave favorable reports from their experience with tetrathionate broth in Montevideo, as did Varela[11] in Mexico.

In the Miami branch of the Florida State Health Laboratories, 324 fecal samples yielded ten Salmonellas when tetrathionate broth was used; previously, with selenite-F broth, 472 specimens were examined and no Salmonellas were found.

In the examination of 8,093 stools in Florida's Central Laboratory using tetrathionate, Galton and Quan found: 1.32 per cent Salmonellas, 0.97 per cent typhoid, and 1.03 per cent dysentery bacilli. Formerly when selenite-F was used, they had encountered 0.5 per cent Salmonellas, 2.82 per cent typhoid, and 1.38 per cent dysentery bacilli. The decline in typhoid isolations was partly due to the rapid decline in typhoid cases in the state, but it was evident that tetrathionate did not aid them in isolation of either *typhosus* or *dysenteriae*, though the broth was extremely sensitive for Salmonellas.

Tetrathionate broth was used in the study of a *Salmonella typhimurium* outbreak among premature infants by Watt and Carlton.[14] Of seventy-seven positive cultures, twenty-nine were found by direct platings on SS, fifty-six were found only through tetrathionate enrichment broth.

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Received for publication, Dec. 14, 1949.
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METHODS

Most specimens were received as rectal swabs; a few were stools. All were streaked directly on EMB (eosin-methylene blue) and SS (Salmonella-Shigella) plates, then inoculated into Difco's tetrathionate broth. Rectal swabs were left in the tetrathionate. The plates were inoculated by rubbing the specimen over a quarter-sized area of an SS plate and an EMB plate. A second EMB plate was inoculated by streaking with the loop which had been used to streak the first plate of this medium. This resulted in one EMB plate with a heavy inoculum and one with a light inoculum, necessary since the number of organisms in feces cannot be anticipated. Because SS is more inhibitive to the coli group than EMB, only one plate of this is required.

The next day colonies were picked to TSI* slants. The tetrathionate broth was streaked to one plate of SS medium. Slants of TSI with suggestive reactions were studied with fermentation reactions and sera. When a specimen had a large number of suspicious colonies on EMB plates, they were set up directly with sera, without waiting for their reactions on slants. Antisera used consisted of Lederle's antisera to members of the Shigella genus and a polyvalent antiserum for Salmonella O antigens. Species identification of Salmonella was not made in our laboratory.†

TABLE I. MEDIUM FROM WHICH ISOLATIONS WERE MADE

	DIRECT PLATES ONLY	TETRATHIONATE BROTH ONLY	BOTH DIRECT PLATES AND TETRATHIONATE BROTH
Eberthella typhi			1
Flexner			27
Sonnei	4	3	22
Alkalescens	1		3
Dispar			1
Salmonellas	1	14	25
Friedländer			1
Proteus rettgeri		2	
Paracolon bethesda		4	2

The results are tabulated in Table I and summarized in Table II. It is evident that about a fifth of the total positives would have been missed had we not supplemented plates with tetrathionate broth. Thirty-seven per cent of the Salmonellas would have been unrecognized had we not used this enrichment broth. The isolation of Shigella organisms was not correspondingly fostered, but in our experience tetrathionate was only occasionally inhibitive to the Shigellas.

TABLE II

Total specimens	740
Negative	629
Positive	111
Positives found on direct plates only	6
Positives found by tetrathionate broth only	23
Positives found both on direct plates and from tetrathionate broth	82

DISCUSSION

The increase in Salmonella isolations which tetrathionate broth made possible for us is definitely worth while but is small in comparison with the 700 per

*Triple Sugar Iron Agar is prepared by the Baltimore Biological Laboratory.

†We wish to express our gratitude to Dr. MacDonald Fulton, Dr. O. O. Felsenfeld, and Dr. P. R. Edwards who have identified many isolates for us.

cent reported by Kauffmann and the increase also reported by others. We believe this is due to two reasons. (1) We had the advantage of using the highly selective SS medium for direct platings. (2) Our work involved a high percentage of acute cases. Tetrathionate enrichment is most valuable for detecting the small numbers of Salmonella found in convalescents and carriers.

The rettgeri-like organisms are of interest for several reasons. Such an organism was found repeatedly in stool cultures in a fatal case of diarrhea in a 6-month-old child, suggesting that this organism—the causative agent in fowl typhoid—may at times possess a marked toxicity for infants. The second child in whom a Rettger's bacillus was found suffered diarrhea but recovered. Salmonella infection in man has been traced to the meat of chicken eggs by Watt.[13] To date, *Proteus rettgeri* has not been recovered from the interior of chicken eggs.

The strains of Rettger's bacillus which we isolated had the following characteristics: acid but no gas in glucose; negative in adonitol, dulcitol, lactose, maltose, mannite, sucrose, and xylose; imvic ++−+, urea +, sulfide −. They were agglutinated by an 0 serum for Rettger's bacillus but not by H.

Felsenfeld and Young[4] are among those who have made isolations of Rettger's bacillus from human sources, and Fulton and Curtis[5] have recently published a study of this group of organisms.

The organisms described as *Paracolon bethesda* are ones agglutinated with antiserum 100 $77A_1$ or 100 $90B_2$. Such a bacterium was first described by Barnes[1] in an outbreak of diarrhea in the Navy in 1946. Of the six patients in our series who harbored this bacillus in the stools, four had diarrhea, one had weight loss with no diarrhea, and one had virus pneumonia without diarrhea. In addition to the six strains found in the stools, one isolation was made from a gall bladder. Seligmann, Saphra, and Wassermann[10] have reported the occurrence of Salmonella in the gall bladder, sometimes causing fatal infections. In the present case, the patient had a history of chronic cholecystitis of six years' duration; the paracolon was recovered from the bile after surgical removal of the gall bladder. Our small number of cases adds little evidence concerning the pathogenicity of *Paracolon bethesda*. More data are included in a paper by Beene, Bohls, and Fulton.[2] Our work does suggest that tetrathionate is of great benefit in isolating the organism, whatever is its significance.

The organism which we have designated as Friedländer's bacillus was isolated from a child suffering from diarrhea, dehydration, and congenital syphilis. The bacterium has at least moderate pathogenicity as indicated by the fact that when 0.1 c.c. of a 1 to 1000 dilution of a sixteen-hour broth culture was inoculated intraperitoneally into a mouse, the animal succumbed in four days. The organism was then recovered in large numbers from the peritoneal fluid and heart's blood. This was not a Type A, B, or C, Friedländers. Walcher[12] has reported an epidemic in a nursery in which Friedländer's was isolated from the stools of the babies. We do not know of any evidence that special media help in the isolation of this bacterium.

Flexner types isolated in our work consisted of one type 1, three type 2, five type 3, two type 4, one type 5, eleven type 6, three type 8, and one type 13. We noted the crossing of Flexner type 1 with anti-Salmonella serum as reported by Ewing and Bruner.[3]

Our figures include work for the months of April to November inclusive. Beginning in December, positive stools ceased to be found, except in a rare carrier, although diarrheas of presumably virus origin increased.

SUMMARY

A 37 per cent increase in Salmonella isolations resulted by the use of tetrathionate broth compared with isolations from plates alone.

Tetrathionate also proved of value in isolating Paracolon 100 $77A_1$ and 100 $90B_2$ (*bethesda*) and organisms related to Rettger's bacillus.

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EFFECT OF HIGH SERUM CONCENTRATION ON THE GROWTH OF BACILLUS CEREUS IN BROTH

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DORNBUSH and Pelcak* have used *Bacillus cereus* (strain B, 5) for the rapid determination of aureomycin levels in serum and other body fluids. The following experiment was undertaken to determine whether serum alone in high concentration was inhibitory to the organism, thus giving rise to erroneous results in the aureomycin sensitivity tests.

EXPERIMENT

The sera used were placed in small culture tubes in 1 c.c. amounts. The first tube of each series consisted of undiluted serum. The second to fifth tubes were serially diluted with sterile Trypticase soy broth (Baltimore Biological Laboratory). This gave serum dilutions ranging from undiluted to 1:8. Human, sheep, horse, cow, and rabbit sera were tested. The culture of *B. cereus* was grown in Trypticase soy broth for eighteen hours. This eighteen-hour culture was diluted 1:10 with sterile broth and 0.1 c.c. was added to each tube of the sera as prepared above. A control tube included in the series contained broth and no serum. The tubes were incubated for four hours in the 37° water bath and the results recorded.

TABLE I. EFFECT OF HIGH SERUM CONCENTRATION ON THE GROWTH OF BACILLUS CEREUS IN BROTH

SERUM	SERUM DILUTION IN BROTH					
	UNDILUTED	1:1	1:2	1:4	1:8	CONTROL
Human 1	C	D	D	D	D	D
Human 2	C	D	D	D	D	D
Human 3	C	D	D	D	D	D
Human 4	C	D	D	D	D	D
Human 5	C	D	D	D	D	D
Human 6	C	D	D	D	D	D
Horse 1	C	C	D	D	D	D
Horse 2	C	C	D	D	D	D
Horse 3	C	C	D	D	D	D
Horse 4	C	C	D	D	D	D
Horse 5	C	C	D	D	D	D
Horse 6	C	C	D	D	D	D
Sheep 1	C	C	D	D	D	D
Sheep 2	C	C	D	D	D	D
Cow 1	C	C	C	D	D	D
Cow 2	C	C	D	D	D	D
Rabbit 1	C	D	D	D	D	D
Rabbit 2	C	D	D	D	D	D
Rabbit 3	C	D	D	D	D	D
Rabbit 4	C	D	D	D	D	D
Rabbit 5	C	D	D	D	D	D
Rabbit 6	C	D	D	D	D	D

C, Clumped with sediment on bottom of tube.
D, Diffuse growth.

From The Wilmer Ophthalmological Institute of the Johns Hopkins Hospital and University.

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*Dornbush, A. C., and Pelcak, E. J.: The Determination of Aureomycin in Serum and Other Body Fluids, Ann. N. Y. Acad. Sc. 51: 218, 1948.

RESULTS

No inhibition of growth occurred in any of the sera tested. It was noted however that there was a marked difference in the type of growth obtained. In the tubes containing undiluted sera the growth was in the form of clumps which settled to the bottom of the tube, giving a clear supernatant, which gave the appearance of no growth being present at first glance. In the case of some sera this phenomenon was more marked, being observed to occur up to a dilution of 1:2 (Table I).

SUMMARY AND CONCLUSION

1. *B. cereus* (strain B, 5) is not inhibited by high serum concentrations.

2. *B. cereus* in broth culture containing high concentrations of serum grows in clumps which settle to the bottom. This fact should be borne in mind and the tubes vigorously shaken before the readings are made when this organism is used in the determination of antibiotic levels.

A SIMPLE METHOD FOR ROUTINE ASSAY OF AUREOMYCIN IN BODY FLUIDS

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TO BE clinically effective in the treatment of infectious diseases, dosage schedules for antibiotics must be capable of achieving and maintaining therapeutic levels in body fluids. In actual practice therapeutic levels should approximate the in vitro sensitivity value of the infecting organism.

Our procedure for determining streptomycin levels in body fluids[1] has proved consistent, practical, and satisfactory for routine use. This procedure was modified and adapted for estimating aureomycin by utilizing as standard an aureomycin-sensitive organism grown in an appropriate medium.

The basic concept of the proposed aureomycin assay method calls for the most simple, least-enriched medium which will insure adequate growth of the test organism *Bacillus cereus*. The medium selected for level determinations, although relatively deficient, must still compare more or less favorably in growth-promoting properties with routine culture media. The test organisms, when grown in culture media with minimal growth factors, are rendered more susceptible to the action of aureomycin, thus making the assay method more sensitive. This, by permitting higher dilutions of body fluids, eliminates or reduces (a) the growth stimulus of such fluids and (b) any serologic interference due to contained native antibodies.

METHODS

The media which were investigated were: Amigen (Mead Johnson & Co.), Bacto-Peptone (Difco), Bacto-Tryptone (Difco), Bacto-Tryptose (Difco), Neo-Peptone (Difco), N-Z Case (Sheffield Farms Co., Inc.), Soy Bean Hydrolysate (The Arlington Chemical Company), and Yeast Extract (Difco).

These media were made up in 1 per cent concentrations and carefully filtered. A clear broth is essential for dependable readings. The media are autoclaved for twenty minutes at 15 pounds pressure. All glassware must be extremely clean, since soap residue has been found to counteract the potency of the antibiotic.

*B. cereus** grows luxuriantly in simple media and is readily inhibited by aureomycin. It is therefore ideally suited as a test organism.[2] It was found that *B. cereus* grows luxuriantly in a variety of mediums at the optimum concentrations noted in the last line of Table I.

The growth curves were determined in twelve test tubes containing serial twofold dilutions of each medium tested. Sterile water was used as diluent. The broth concentrations in the tubes ranged from 1 per cent to 0.0005 per cent in 2 ml. amounts. The test organisms were grown overnight at 37° C. and diluted 10^{-3} in sterile water and added in 0.1 ml. amounts. The stock culture is kept on nutrient agar in the refrigerator at 5° C. All tubes were incubated for eighteen hours and read turbidimetrically for presence and density of growth.

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*The strain of *B. cereus* used in our tests was kindly supplied by Lederle Laboratories Division, American Cyanamid Company.

The range of growth was designated as none (0), retarded (1 plus), and dense (4 plus). The results are recorded in Table I.

The sensitivity of *B. cereus* to aureomycin varies in simple media in accordance with the concentration. To study the sensitivity in the various media investigated, serial twofold dilutions were set up in 1 ml. amounts containing 1 per cent, 0.5 per cent, 0.25 per cent, and 0.125 per cent of test broth. The content of aureomycin used in this experiment varied from 0.5 μg to 0.00025 μg per milliliter test broth. Each tube was seeded with an overnight culture diluted 10^{-4} in 1 ml. amounts, making a total of 2 ml. per tube. All tubes were incubated for eighteen hours and read turbidimetrically for presence or inhibition of growth. The controls in each instance supported good growth.

TABLE I. GROWTH OF B. CEREUS IN DIFFERENT BROTH MEDIA OF VARIOUS CONCENTRATIONS

	MEDIA							
GROWTH	AMIGEN (%)	BACTO-PEPTONE (%)	BACTO-TRYPTONE (%)	BACTO-TRYPTOSE (%)	NEO-PEPTONE (%)	N-Z CASE (%)	SOY BEAN HYDROLYSATE (%)	YEAST (%)
None (0)	.001	.002	.001	.001	.0005	.001	.002	.001
Retarded (1+)	.002	.016	.016	.016	.004	.016	.004	.004
Dense (4+)	.063	.5	.5	.5	.5	1.	.5	.25

The results, as tabulated in Table II, indicate that media deficient in nutritional value generally render *B. cereus* more sensitive to aureomycin. The antibiotic effect is inversely proportional to the concentration of the medium.

Of the group of media investigated, we finally selected as standard Soy Bean Hydrolysate in a final concentration of 0.5 per cent. Besides sustaining adequate growth of *B. cereus*, it has the added advantage of not becoming contaminated as readily as other media which were studied.

TABLE II. AUREOMYCIN SENSITIVITY OF B. CEREUS IN DIFFERENT BROTH MEDIA OF VARIOUS CONCENTRATONS

	MEDIA							
CONCENTRATION MEDIA (%)	AMIGEN	BACTO-PEPTONE	BACTO-TRYPTONE	BACTO-TRYPTOSE	NEO-PEPTONE	N-Z CASE	SOY BEAN HYDROLYSATE	YEAST
	INHIBITORY LEVEL (μG AUREOMYCIN/ML. BROTH)							
1	.012	.016	.032	.032	.16	.063	.008	.045
.5	.008	.016	.032	.024	.012	.032	.005	.032
.25	.008	.012	.024	.024	.012	.032	.004	.016
.125	.008	.008	.024	.016	.012	.032	.004	.016

Whole blood obtained by venipuncture is promptly centrifuged; the serum is pipetted off and stored in the deep freezer until used for assay. *B. cereus* is sensitive to 0.005 μg of aureomycin per milliliter of 0.5 per cent Soy Bean Hydrolysate, and since our test calls for 2 ml., as little as 0.01 μg per milliliter serum can be detected. Levels within the range of 1 to 10 μg require 1:10 dilution. To simplify the technique, all sera may be diluted in sterile water. They are pipetted into seven 100 by 13.5 mm. tubes as follows: 0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., 0.6 ml., 0.8 ml., 1 milliliter.

Sterile water is added to all tubes sufficient to make 1 ml. of serum dilution. One milliliter of 1 per cent Soy Bean Hydrolysate seeded with *B. cereus* is added to all tubes, making the final concentration of the broth 0.5 per cent. The seeded broth is prepared by diluting an eighteen-hour culture 10^{-4}.

A standard containing 0.2 μg of aureomycin per milliliter is diluted ten times and set up in 0.1 ml. to 1 ml. amounts and treated the same way as the unknown serum.

All tubes are incubated at 37° C. for eighteen hours and read turbidimetrically for growth or inhibition. The values are calculated in micrograms aureomycin per milliliter serum.

Under the conditions of the test, 0.01 μg aureomycin per milliliter serum is required to inhibit growth of *B. cereus* in 0.5 per cent Soy Bean Hydrolysate.

DIAGRAM OF TEST

Amount of serum	.1	.2	.3	.4	.6	.8	1.
Amount of water	.9	.8	.7	.6	.4	.2	0

To each tube add 1 ml. Soy Bean Hydrolysate 1% broth seeded with *B. cereus* (18-hour culture diluted 10^{-4})

Amount of standard	.1	.2	.3	.4	.5	.6	.7	.8	.9	1.
Amount of water	.9	.8	.7	.6	.5	.4	.3	.2	.1	0

CALCULATION

$$\frac{\text{Sensitivity of test organisms}}{\text{Amount of serum inhibiting } B.\ cereus} \times \text{dilution factor} = \mu\text{g aureomycin/ml. serum.}$$

If 0.3 ml. serum diluted 1:100 inhibits the growth of *B. cereus* in 0.5 per cent Soy Bean Hydrolysate, the serum contains 3.33 μg aureomycin per milliliter serum.

$$\frac{0.01}{0.3} \times 100 = 3.33\ \mu\text{g aureomycin/ml. serum.}$$

SUMMARY AND CONCLUSIONS

A simple, practical method for assay of aureomycin in body fluids is presented. This test is similar in concept and technique to our method for estimating streptomycin in body fluids. The tests differ only in the media and test organisms employed.

B. cereus was adopted as test organism. It has been demonstrated that *B. cereus* grows very readily in simple media, deficient in growth factors, which renders the test organism proportionately more sensitive to aureomycin. Minute amounts of the antibiotic can thereby be determined.

It is possible to measure routinely the extent and duration of aureomycin levels in body fluids. In this manner dosage schedules can be planned more effectively.

The clinical application of these studies will be made the subject of a subsequent report.

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